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(54) Title: HYBRID PHOSPHOINOSITIDE PHOSPHOLIPIDS: COMPOSITIONS AND USES

(57) Abstract: The methods and compositions disclosed herein concern the synthesis of a novel class of "two-headed" phospholipid-phosphoinositide hybrids possessing a carbon backbone, such as 2,3-diacylthreitol, erythritol or a synthetic module. The second phospholipid head group allows introduction of a biochemical or chemical moiety in a position orthogonal in space to those occupied by the phosphoinositide head group and the two acyl chains. The diacyl moieties allow for the incorporation of Pea-PIP<sub>2</sub> into a lipid bilayer, while the PtdIns(4,5)P<sub>2</sub> moiety in the aqueous layer is specifically recognized by lipid binding proteins. In alternative embodiments of the invention, reporters, for example biotin, fluorophores and/or spin labels, are attached to the free amino group of the head groups of such molecules to specifically target the reporters to the lipid-water interface.

## HYBRID PHOSPHOINOSITIDE PHOSPHOLIPIDS: COMPOSITIONS AND USES

### CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority from U.S. Provisional Patent Application  
Numbers 60/368,556 filed March 29, 2002 and 60/392,783 filed June 28,  
2002.

### STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

10 The U.S. Government has certain rights in this invention based upon  
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### BACKGROUND OF THE INVENTION

15 Phosphoinositides ("PtdInsP<sub>n</sub>s") are biosynthesized by the interplay of  
kinases and phosphatases. These charged lipids are minor components of  
cellular membranes but are vital as second messengers for diverse cellular  
functions. PtdInsP<sub>n</sub>s are essential elements in tyrosine kinase, growth factor  
receptor and G-protein receptor signaling pathways. Furthermore, these lipid  
signals have important roles in membrane trafficking, including endocytosis,  
20 exocytosis, Golgi vesicle movement and protein trafficking, in cell adhesion  
and migration, in remodeling of the actin cytoskeleton, and in mitogenesis and  
oncogenesis. Activation of cellular signaling pathways often results from  
production of one of eight specific PtdInsP<sub>n</sub>s in response to a stimulus, and  
each PtdInsP<sub>n</sub> has a specific role for a given signaling pathway in each  
25 cell-type.

Phosphoinositide recognition by binding proteins and lipid-metabolizing  
enzymes involves specific interactions with the phosphoinositide head group  
and diacylglycerol backbone which vary significantly from protein to protein.  
When a fluorescent probe is introduced into the inositol head group, binding  
30 and metabolism can be attenuated or abrogated entirely. Moreover, many  
acyl-modified phosphoinositides fail to show adequate  $K_m$  and  $V_{max}$  values as  
substrates for lipid kinases and phosphatases. Further, certain  
phosphoinositide binding proteins demonstrate reduced binding to head

group- or acyl-modified phosphoinositides. The simple, robust assays needed for biochemical and cellular studies require a chemically-modified phosphoinositide substrate that can be both acted on by enzymes and recognized by specific binding proteins. A need exists for novel types of 5 phosphoinositides derivatives whose modifications are consistent with the natural binding affinities and sub-cellular localization of the native compounds. Derivatives of this sort would also function as tools useful in drug discovery or drug development assays, and for basic research.

10

## SUMMARY OF THE INVENTION

The present methods and compositions relate to the fields of pharmacology and drug discovery. More particularly, the methods and compositions concern derivatives of phosphatidylethanolamine-extended phosphoinositides and phosphoinositide polyphosphates (PtdInsP<sub>n</sub>s) and use 15 thereof in drug discovery and development of assays, as well as for basic research purposes. The present invention concerns the design and asymmetric total synthesis of the first examples of a new class of functionalized PtdInsP<sub>n</sub>s, the Pea-PIP<sub>n</sub>s.

The synthetic strategy involves homologation of the 1,2-diacylglycerol 20 backbone to a carbon threitol backbone, such as 2,3-diacylthreitol, erythritol or synthetic module. As seen in Figure 1, such hybrid lipids possess a phosphatidylethanolamine (PE, or Pea) head group at the 1-position and a PtdInsP<sub>n</sub> head group at the 3,4 and/or 5-position. A reporter group, for example biotin, a fluorophore, or a spin label, may then be covalently attached 25 to the free Pea amino group. Figure 1 shows an unmodified dipalmitoyl PtdIns(4,5)P<sub>2</sub> at center, with the acyl-modified NBD (fluorescent *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)) derivative above and an exemplary Pea-PIP<sub>n</sub> NBD derivative at the bottom. The reporter groups in these synthetic constructs are targeted to the lipid-water interface at a site distant from the 30 key PtdInsP<sub>n</sub> head group recognition features. The unchanged diacyl moiety permits insertion and retention of Pea-PIP<sub>n</sub>s in a lipid bilayer to facilitate recruitment of PtdInsP<sub>n</sub>-specific binding proteins to a membrane surface environment.

These new hybrid lipids can serve as direct enzymatic substrates that can be delivered into a cell in order to measure direct turnover. This is a great improvement over measuring competitive displacement with a surrogate. Accordingly, Pea-PIP<sub>n</sub>s of the present invention have potential for the 5 production of unique, reporter-based high throughput screens or assays for *in vitro* biochemical activity and for monitoring real time *in situ* biochemical activity in living cells.

#### BRIEF DESCRIPTION OF THE FIGURES AND DRAWINGS

10 The following figures form part of the present specification and are included to further demonstrate certain aspects of the disclosed embodiments of the invention. The embodiments of the invention may be better understood by reference to one or more of the figures in combination with the detailed description of specific embodiments of the invention presented herein.

15 Figure 1. Illustrates (at c) an exemplary hybrid lipid of the present invention that possess a phosphatidylethanolamine (PE, or Pea) head group at the 1-position and a PtdIns(4,5)P<sub>2</sub> head group at the 4-position in accordance with one embodiment of the present invention. Shown are (a), the unmodified Dipalmitoyl-PtdIns(4,5)P<sub>2</sub> at the center; (b), the acyl-modified 20 NBD-derivative above (1-C<sub>6</sub>-NBD,2-C<sub>6</sub>-PtdIns(4,5)P<sub>2</sub>); and (c), the Pea-PIP<sub>n</sub> NBD derivative at the bottom (NBD-Pea- PI(4,5)P<sub>2</sub>).

25 Figure 2. Illustrates synthesis of exemplary Pea-PIP<sub>n</sub>s, including protection debenzylation by hydrogenolysis resulting in each of eight desired Pea-PIP<sub>n</sub>s.

30 Figure 3. Illustrates exemplary synthesis of differentially functionalized 2,3-diacylthreitol backbones in accordance with an embodiment of the present invention. Reagents and conditions: (a) cyclopentanone, *p*TSA, toluene, reflux; (b) LiAlH<sub>4</sub>, THF; (c) NaH, *p*-methylbenzyl (PMB)Cl, DMF; (d) 1-*H*-tetrazole, Cbz-aminoethyl phosphoramidite 4, CH<sub>2</sub>Cl<sub>2</sub>; (e) 1 M HCl,

tetrahydrofuran (THF); (f) C<sub>15</sub>H<sub>31</sub>COOH, dicyclohexylcarbodiimide (DCC), DMAP, CH<sub>2</sub>Cl<sub>2</sub>; (g) dichlorodicyanoquinone (DDQ), CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O.

Figure 4. Illustrates exemplary backbone phosphorylation and synthesis 5 of Pea-PIP<sub>2</sub> derivatives **12**. Reagents and conditions: (a) BnOP(NiPr<sub>2</sub>)<sub>2</sub>, 1-H-tetrazole, CH<sub>2</sub>Cl<sub>2</sub>; (b) 1-H-tetrazole, 4,5-HG, CH<sub>2</sub>Cl<sub>2</sub>; (c) H<sub>2</sub> (60 psi), 10% Pd/C, THF/H<sub>2</sub>O; (d) probe-NHS ester, 0.5 M TEAB, DMF.

Figure 5. Illustrates exemplary Pea-PIP<sub>n</sub> reporter groups according to an 10 embodiment of the present invention. Reaction of the free Pea amino group of with four N-hydroxysuccinimidyl (NHS) esters afforded the corresponding biotinylated derivative 4a, the fluorescent *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) and 6-carboxyfluorescein derivatives 4-b and 4-c, and the spin-labeled 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXYL) derivative 4-d.

15 Figure 6. Illustrates an exemplary linker-modified Pea-PIP<sub>n</sub> analog wherein the linker modification is an amino-PEG-amide.

20 Figure 7. Illustrates the synthesis of exemplary short PEG linkers.

Figure 8. Illustrates the synthesis of an exemplary protected threitol backbone with PEG linkers.

#### DETAILED DESCRIPTION

25 One embodiment of the present invention comprises a strategy for synthesizing a novel class of "two-headed" phospholipid-phosphoinositide hybrids possessing a carbon threitol backbone, such as a 2,3-diacylthreitol, erythritol or synthetic module. These hybrid lipids possess a phosphatidylethanolamine (PE or Pea) head group at the 1-position and a 30 PtdInsP<sub>n</sub> head group, such as PtdIns(4,5)P<sub>2</sub>, at the 3,4 and/or 5-position. In particular embodiments, a reporter group, e.g., biotin, a fluorophore, or a chelating agent, may then be covalently attached to the free Pea amino

group. The reporter would thus be targeted to the lipid-water interface at a site distant from the key PtdInsPn head group (for example, PtdIns(4,5)P<sub>2</sub>) recognition features of the binding protein. In various embodiments, the diacyl moiety permits insertion and retention of Pea-PIPns in a lipid bilayer to 5 facilitate recruitment of PtdInsPn head group- (for example, PtdIns(4,5)P<sub>2</sub>-) specific binding proteins to a membrane surface environment.

The additional phospholipid head group allows introduction of a biochemical or chemical moiety in a position orthogonal in space to those occupied by the phosphoinositide ("PIP<sub>n</sub>") head group and the two acyl 10 chains. The method for producing the hybrid phospholipids of the present invention involves diethyl D-tartrate as the chiral precursor for the extended glycerol backbone of the target hybrid lipid. The corresponding acetal is reduced with lithium aluminum hydride and protected with 1 equivalent of PMB-Cl to give the monobenzyl ether. Coupling with a carbonylbenzyloxy 15 (Cbz)-protected aminoethoxy phosphoramidite yields, after oxidation, the protected phosphatidyl analogue ready for addition of the acyl chains and the selected PIP<sub>n</sub> head group. After acetal hydrolysis, the 2,3-dipalmitoyl derivative is prepared. As shown for exemplary molecules in Figure 2, the 20 functionalized carbon threitol backbone is then coupled with a phosphatidyl head group. Debenzylation by hydrogenolysis then affords, in particular embodiments, each desired phosphatidylethanolamine -phosphoinositide ("Pea-PIP<sub>n</sub>") with a free amino groups ready for derivatization.

Any PIP<sub>n</sub> head group may be prepared and used according to the invention. PIP<sub>n</sub> head groups to be utilized in this invention may be 25 commercially obtained or prepared by standard methods known in the art.

In a particular embodiment of the invention, members of this class of molecules have a phosphatidylethanolamine ("Pea") head group at the 1-position and a phosphoinositide ("PIP<sub>n</sub>") head group at the 3,4 and/or 5-position.

According to this embodiment, the Pea-diacylthreitol synthetic module 30 is prepared and coupled with any selected phosphoinositide head group. For example, each of eight different naturally occurring phosphoinositide head groups (PI, PI(3)p, PI(4)P, PI(5)P, PI(4,5)P<sub>2</sub>, PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>)

has been used to produce Pea-PIP<sub>n</sub>s of the present invention (the head groups used for synthesizing Pea-PIP<sub>n</sub>s are available commercially, for example from Echelon Biosciences Inc., SLC Utah). Accordingly, certain 5 embodiments of the present invention include Pea-PI, Pea-PI(3)P, Pea-PI(4)P, Pea-PI(5)P, Pea-PI(3,4)P<sub>2</sub>, Pea-PI(3,5)P<sub>2</sub>, Pea-PI(4,5)P<sub>2</sub> and Pea-PI(3, 4,5)P<sub>3</sub>.

In another embodiment of the present invention, a linker may be utilized between the PIP<sub>n</sub> headgroup and a reporter. In a particular embodiment, the length of the linker between the PIP<sub>n</sub> headgroup and the 10 reporter moiety at the primary amine is lengthened. In a particular embodiment, the extension for the linker comprises an oligo-polyethylene glycol linker. According to this embodiment, a commercially available di-, tri-, tetra-, and/or penta(ethylene glycol) is used for extending the linker during synthesis of the Pea-PIP<sub>n</sub>s.

15 Other embodiments include additional or alternative linkers. For example, one such alternative linker is a diamino linker that will yield a phosphoramidate final product rather than a phosphate linkage. Another alternative linker uses both phospho- and non-phospho linked spacers. In certain embodiments, this can be accomplished by replacing the Pea group at 20 C-4, such as with a simple ester, amide or other linkage that allows a pendant functionality to be incorporated. Another alternative linker is to use a phosphatidylserine or other carboxylic acid instead of Pea to permit further functionalization at the end distal to the PIP<sub>n</sub> recognition element. Various methods for producing alternative linkers are well known in the art.

25 Pea extension of the invention may also include aminoalcohols as linkers, for example 3-aminopropanol, 4-aminobutanol, and others. In certain embodiments heteroatom-containing derivatives such as 1-amino-11-hydroxy-3,6,9-triaundecane or similar aminoalcohols with water soluble spacer chains may be used. These can include branched aminoalcohols including, but not 30 limited to, for example, 2-aminomethyl-3-amino-1-propanol which has multiple reactive amino termini for addition of two or more biochemical probes. One of ordinary skill in the art will know methods of Pea extension beyond those listed above.

According to certain embodiments of the present invention, the acyl chains to be attached to any of the above mentioned head groups may include any acyl group from n=2 to n=26 carbons. Methods of modifying the length or degree of double bonds in an acyl chain are well known in the art. In 5 certain embodiments, the carbons will have a number of double bonds, for example from 0 to 6. In alternative embodiments, one or both acyl chains can be replaced with an ether chain of the same length, degree of unsaturation or terminal functionalization. Replacement of one or both acyl chains can be accomplished by any standard method known in the art.

10 According to other embodiments, the phosphate groups of this invention can be chemically modified to increase stability or resistance to chemical or enzymatic hydrolysis. In certain embodiments, the phosphate groups will be on the inositol, the phosphodiester or the PE phosphodiester. In alternative embodiments, this involves a P=S or P-S bond, replacement of 15 a P-O phosphate linkage with a P-CH<sub>2</sub>, P-CHF or P-CF<sub>2</sub> phosphonate linkage or a phosphoramidate linkage. Other methods of chemically modifying the phosphate groups of this invention are known in the art.

20 In certain embodiments, a Pea-PIP<sub>n</sub> of the present invention has a triester analog at P-1 to allow for an additional site for derivation. Methods of synthesizing a Pea-PIP<sub>n</sub> with a triester analog at P-1 are well known in the art. (See, e.g., Q.-M. Gu and G.D. Prestwich, "Synthesis of Phosphotriester 25 Analogues of the Phosphoinositides PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>," *J. Org. Chem.*, 61, 8642-8647 (1996)).

25 In yet another embodiment, the Pea-PIP<sub>n</sub> carbon threitol backbone can include four, five, six or more carbons. In particular embodiments, such carbon backbone may be branched. In a certain embodiment, such carbon threitol backbone is 2,3-diacylthreitol or erythritol.

30 In yet another embodiment, Pea-PIP<sub>n</sub>s include a polymerizable group that allows for the construction of Pea-PIP<sub>n</sub>s polymers. Accordingly, particular embodiments of the invention comprise oligomeric Pea-PIP<sub>n</sub>s formed by linking two or more Pea-PIP<sub>n</sub> molecules together.

In particular embodiments, a reporter group (or "label" or "tag") is covalently attached to the free Pea amino group. Such a reporter may

include, for example, a fluorescent label, a radiolabel, a chemiluminescent label, a spin label, a photophore, a chromophore, biotin, a nanogold particle, and/or any suitable reporter, and mixtures thereof.

As used herein, suitable fluorescent compounds that can be used  
5 according to the present invention include chemically activated, thetherable  
analogs of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl,  
dialkylaminocoumarin, eosin, erythrosine, fluorescein (FITC),  
hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol  
Green, TMR, Texas Red, X-Rhodamine, and the like.

10 Attached to the Pea amino group, the reporter would thus be targeted  
to the lipid-water interface at a site distant from the specific features of the  
PtdInsP<sub>n</sub> head group necessary for interaction with lipid recognition proteins  
or other chemicals or compounds that specifically interact with the PtdInsP<sub>n</sub> at  
a membrane surface. In various embodiments, the diacyl moiety permits  
15 insertion and retention of Pea-PIP<sub>n</sub>s in a lipid bilayer to facilitate recruitment of  
PtdInsP<sub>n</sub>-specific binding proteins to a membrane surface environment. An  
individual Pea-PIP<sub>n</sub> may have one or more reporters which may be of the  
same or different types. In various embodiments, the Pea-PIP<sub>n</sub>s will be  
transported into cells, with or without a reporter.

20 All stereoisomers, which include enantiomers or diasteromers, for any  
component of the Pea-PIP<sub>n</sub> molecules can be employed in any embodiment  
of this invention. Such modifications are well known in the art.

Certain embodiments will include Pea-PIP<sub>n</sub>s bound to a surface, for  
example for use in a biochemical assay. In particular, embodiments, such  
25 surface will include a plate, a bead or nitrocellulose. In a particular  
embodiment, such surfaces are selected from the group consisting of, but not  
limited to, a chemically activated glass, plastic or other surface; activated  
agarose, polystyrene or any other type of bead and nitrocellulose. In other  
embodiments, the Pea-PIP<sub>n</sub>s are attached to a metal surface, such as gold.  
30 In particular embodiments, attachment to gold is accomplished by introducing  
to the Pea-PIP<sub>n</sub> a pendant alkyl thiol moiety that is capable of attaching to a  
gold surface. Methods for making such pendant alkyl thiol derivatives are well  
known in the art.

In alternative embodiments, the Pea-PIP<sub>n</sub>s will be incorporated in a liposome. Such liposome incorporated Pea-PIP<sub>n</sub>s may include a reporter.

5 Certain embodiments allow for the use of Pea-PIP<sub>n</sub>s for assays. The compositions and methods of their use can be used in any assay that currently uses a modified Pea-PIP. Particular embodiments include *in vitro* fluorogenic, FRET, ELISA and chemiluminescence assays. These may be in a high-throughput format. Alternative embodiments include *in vitro* enzyme assays, lipid kinase or phosphatase activity, cell-based assays and agonist or antagonist assays.

10 Such assays include, but are not limited to, *in vitro* enzyme assays, *in vitro* agonist or antagonist assays or cell-based assays. In some embodiments of the invention, labeled Pea-PIP<sub>n</sub>s can also be linked or bound to plates, beads or other surfaces which may, in particular embodiments, be coated with a means for binding Pea-PIP<sub>n</sub> thereto. Labels of use in the 15 present invention may be activated by any method known in the art in order to effect attachment to a Pea-PIP<sub>n</sub> of the present invention. Methods of use in the attachment of a label include, but are not limited to, the activation of an ester, carbonyldiimidazole activation and use of any Michael acceptor, such as acrylates, acrylamide, maleimides, vinylsulfone, a,b-unsaturated ketones, 20 esters, aldehydes, amides, and the like.

25 An example of such a means of linking or binding a Pea-PIP<sub>n</sub>s is streptavidin. Another example is NHS activation. In other embodiments, the Pea-PIP<sub>n</sub> is bound to nitrocellulose. In particular embodiments, the reporter is biotin or a fluorescent label. Fluorescent compounds suitable for use as a label or reporter according to the present invention include, but are not limited to, chemically activated tetherable analogs of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein (FITC), hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, X-Rhodamine, and the like.

30 One embodiment of the present invention provides a method of screening for phosphoinositide-specific binding proteins in a membrane surface environment.

In particular embodiments a high throughput screen (HTS) comprising compositions of the invention can be used for identifying, for example, chemicals, natural products and/or synthetic compounds that affect phosphoinositide recognition and/or signaling at a cell membrane. Such compounds include, but are not limited to, for example, agonists and antagonists for protein kinases and phosphoinositide kinases and for phosphoinositide and inositol phosphate binding proteins that are regulated by PIP<sub>n</sub>s or IP<sub>n</sub>s and may serve as downstream effectors in signaling pathways important for therapeutic interventions. In particular embodiments, lipid phosphatases or phospholipases are identified. In alternative embodiments, HTS assays include cell-based assays using intracellular PIP<sub>n</sub>s introduced by the shuttling system and can use primary cells, immortalized cells, cancer cells, cells transformed with plasmids encoding key enzymes or other proteins, and the like. The assays could also use *in vitro* cell extracts or partially purified or homogeneous proteins.

In other embodiments the Pea-PIP<sub>n</sub>s are introduced into cells. Such introduced Pea-PIP<sub>n</sub>s may be labeled, or tagged, with one or more reporters. In a particular embodiment, fluorescent acyl-modified Pea-PIP<sub>n</sub>s are shuttled into cells where they exhibit subsequent appropriate membrane localization.

In certain embodiments, assays according to the present invention are performed in living cells. Particular embodiments of the invention provide compositions and methods for visualizing the location of labeled phosphoinositides within a cell. A method for facilitating uptake of a Pea-PIP<sub>n</sub>s into a cell comprises contacting the cell with a composition of matter comprising a Pea-PIP<sub>n</sub> and a shuttle, or other method of introducing the Pea-PIP<sub>n</sub> into the cell. Compounds suitable for shuttling Pea-PIP<sub>n</sub>s into a cell can include, but are not limited to, polyamines. Such polyamines can include, for example, aminoglycosidic aminocyclitols (e.g., aminoglycoside antibiotics), synthetic "spherical" dendrimeric polyamines, polybasic nuclear proteins (histones), polybasic polypeptides, lipidic polyamines, polyethyleneimine, steroidal polyamines, and the like, and mixtures thereof. Other polybasic proteins (or polybasic polypeptides) useful for introducing a Pea-PIP<sub>n</sub> into a cell include proteins or polypeptide that contains sufficient lysine, arginine,

and/or histidine residues to complex an anionic ligand, such as an Pea-PIP<sub>n</sub>. The polybasic polypeptide may also contain unnatural or non-protein amino acids, N-acylglycine groups, and any of a known group of amide group replacements known as peptide bond isosteres. In particular embodiments, 5 assays performed in living cells are monitored by high-content screening methods or confocal microscopy. Such *in vitro* assays allow for minimal disruption of the normal cellular environment.

The disclosed compositions and methods of their use can be used for the discovery of new pharmaceutical agents and targets related to PtdInsP<sub>n</sub>s 10 compounds.

The skilled artisan will realize that the chemical modifications listed above are exemplary only and that many variations may be used, depending on the particular type of Pea-PIP<sub>n</sub> to be synthesized.

15

## DEFINITIONS

For the purposes of the present invention, the following terms shall have the following meanings:

“Tags” or “labels” are used interchangeably to refer to any atom, molecule, compound or composition that can be used to identify a Pea-PIP<sub>n</sub> to 20 which the label is attached. In various embodiments of the invention, such attachment may be either covalent or non-covalent. In certain embodiments of the invention, the labels have physical characteristics that facilitate the identification of the label. In non-limiting examples, labels may be fluorescent, phosphorescent, luminescent, electroluminescent, chemiluminescent or any 25 bulky group or may exhibit Raman or other spectroscopic characteristics. It is anticipated that virtually any technique capable of detecting and identifying a labeled nucleotide may be used, including visible light, ultraviolet and infrared spectroscopy, Raman spectroscopy, nuclear magnetic resonance, electron paramagnetic resonance, positron emission tomography, scanning probe 30 microscopy and other methods known in the art.

Moreover, for the purposes of the present invention, “a” or “an” entity refers to one or more of that entity; for example, “a Pea-PIP” or “a Pea-PIP<sub>n</sub>” refers to one or more of the compound or at least one compound. As such,

the terms "a" or "an", "one or more" and "at least one" can be used interchangeably herein. It is also noted that the terms "comprising," "including," and "having" can be used interchangeably.

Furthermore, a compound "selected from the group consisting of" 5 refers to one or more of the compounds in the list that follows, including mixtures (i.e. combinations) of two or more of the compounds.

According to the present invention, an isolated or biologically pure molecule is a compound that has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to 10 which the compound has been purified. An isolated compound of the present invention can be obtained from its natural source, can be produced using laboratory synthetic techniques or can be produced by any such chemical synthetic route.

As used herein, "shuttle" means a compound, polymer, complex, or 15 mixture thereof that facilitates transport of phosphoinositides, inositol polyphosphates, and mixtures thereof into cells. Preferred shuttles comprise polyamines.

## EXAMPLES

It should be appreciated by those skilled in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be 5 considered to constitute the preferred modes for its practice. However, those of skill in the art should appreciate, in light of the present disclosure, that many changes can be made in the specific embodiments disclosed herein which will still obtain a like or similar result without departing from the spirit and scope of the invention.

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Example 1. Synthesis of Functionalized PhosphoinositidePolyphosphates, the Pea-PIP<sub>n</sub>s, and Reporter Analogs:

The general method for synthesis of Pea-PIP<sub>n</sub>s of the present invention is described in Rzepecki, P.W. and Prestwich, G.D.: *J. Org. Chem.* 2002, 15 67(16):5454-60, which is hereby incorporated by reference in its entirety. In the steps disclosed below, the numbers in bold refer to compounds and synthetic intermediates shown in Figures 3, 4 and 5.

Diethyl D-tartrate was chosen as the chiral precursor for the extended glycerol backbone of the target hybrid lipid. The absolute configuration of 20 both stereogenic centers at C-2 and C-3 is identical to the configuration of glycerol *sn*-2 position in naturally-occurring PtdInsP<sub>n</sub>s and in natural Pea. Moreover, the C<sub>2</sub> axis allowed the use of a monoprotection step in the early stages of the synthesis. Synthesis of an exemplary embodiment, Pea-PI(4,5)P<sub>2</sub> and reporter analogs is shown in Figures 3, 4 and 5. Diethyl D-tartrate **1** was protected as a cyclopentylidene acetal, which was found to be 25 most readily removed after backbone functionalization. Initially, an isopropylidene acetal was used, but scale-up of the deprotection led to unsatisfactory yields of the desired intermediate. Reduction of acetal **2** with lithium aluminum hydride afforded (2*R*,3*R*)-O-cyclopentylidene threitol **3a**, which was protected with 1 equiv. of PMB-Cl to give the monobenzyl ether 30 **3b**. The primary alcohol of **3b** was converted to a Cbz-protected Pea head group by coupling to the phosphoramidite **4**, which after oxidation afforded the

protected PE analogue **5**. Acidic hydrolysis yielded diol **6** in 65% yield after silica chromatography. Acylation with palmitic acid provided diester **7**, and oxidative cleavage of the p-methoxybenzyl (PMB) with dichlorodicyanoquinone (DDQ) gave primary alcohol **8**.

5       Figure 4 illustrates the installation of two different phosphorylated head groups on the 2,3-diacylthreitol backbone. Thus, reaction of alcohol **8** with benzyltetraisopropylphosphordiamidite yielded a homologated Pea-like phosphoramidite reagent **9**, which was coupled with the protected *myo*-inositol 4,5-bisphosphate head group obtained as previously described  
10 (Prestwich, G. D.; Chaudhary, A.; Chen, J.; Feng, L.; B. Mehrotra; Peng, J. In *Phosphoinositides: Chemistry, Biochemistry and Biomedical Applications*; Bruzik, K. S., Ed.; American Chemical Society: Washington, DC, 1999; Vol. 818, p 24-37), to give the fully protected Pea-PIP<sub>2</sub> precursor **10**. Global  
15 debenzylation of **10** was accomplished by hydrogenolysis to give the free phosphate monoesters and phosphodiesters in the hybrid lipid Pea-PIP<sub>2</sub> (**11**). Reaction of the free amino group of **11** with four succinimidyl esters afforded the corresponding biotinylated derivative **12a**, the fluorescent NBD and 6-carboxyfluorescein derivatives **12b** and **12c**, and the spin-labeled PROXYL (tetramethyl-1-pyrrolidinyloxy) derivative **12d**. Biological results are described  
20 below for biotinylated derivative **12a** and fluorescent analogue **12c**. The spin-labeled derivative **12d** may be used to probe interfacial protein-lipid interactions in liposomes, in analogy to the use of acyl spin-labeled probes to characterize the MARCKS peptide-PtdIns(4,5)P<sub>2</sub> interaction in liposomes.

25       The steps comprising an exemplary synthetic process resulting in the novel hybrid lipid, Pea-PI(4,5)P<sub>2</sub>, are described in detail as follows:

(2R, 3R)-1,4-Dioxa-spiro[4.4]nonane-2,3-dicarboxylic acid diethyl ester **2**.  
30       Diethyl D-tartrate **1** (1.004 g, 4.87 mmol), cyclopentanone (2.2 mL, 24.35 mmol, 5 equiv.) and *p*-toluenesulfonic acid (93 mg, 0.49 mmol, 0.1 equiv.) were dissolved in toluene (75 mL) and stirred under reflux for 36 h with azeotropic removal of water using a Dean-Stark trap. Upon completion, the reaction mixture was cooled to room temperature (rt) and neutralized with solid NaHCO<sub>3</sub>. Solid salts were filtered off, the filtrate was concentrated *in*

vacuo, and the crude product was purified on  $\text{SiO}_2$  (hexane:acetone 4:1 containing 10% v/v  $\text{Et}_3\text{N}$ ) to give 1.166 g (4.28 mmol, 88%) of acetal **2** as a colorless oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  4.73 (s, 2H), 4.28 (q, 4H,  $J$  = 5.4), 1.94-2.04 (m, 2H), 1.80-1.90 (m, 2H), 1.65-1.76 (m, 4H), 1.32 (t, 6H,  $J$  = 5.4); 5  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  169.67, 123.34, 77.08, 61.85, 36.66, 23.50, 14.15; IR: 2980, 1756, 1337, 1119, 1115, 1023, 460, 453. Anal. Calcd for  $\text{C}_{13}\text{H}_{20}\text{O}_6$ : C, 57.34; H, 7.40. Found: C, 57.34; H, 7.21.

10 **(2R, 3R)-(3-Hydroxymethyl-1,4-dioxa-spiro[4.4]non-2-yl)-methanol 3a.** A solution of diethyl ester **2** (1165 mg, 4.28 mmol, 1 equiv.) in dry tetrahydrofuran (THF) was transferred *via* canula to a suspension of  $\text{LiAlH}_4$  (244 mg, 6.42 mmol, 1.5 equiv.) in dry THF, pre-cooled in brine/ice bath. The reaction mixture was stirred at rt for 24 h and then saturated aq. potassium sodium tartrate was added dropwise to decompose excess hydride reagent. 15 The mixture was stirred for additional 24 h, and then extracted with three portions of  $\text{CH}_2\text{Cl}_2$ . The combined organic phases were dried over  $\text{MgSO}_4$ , concentrated *in vacuo*, and the crude product was purified on  $\text{SiO}_2$  (hexane:acetone 3:2 containing 10% v/v  $\text{Et}_3\text{N}$ ) to give 805 mg (4.27 mmol, 99%) of the diol **3a** as a colorless oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.85-3.95 (m, 2H), 3.60-3.80 (m, 4H), 1.73-1.85 (m, 4H), 1.60-1.73 (m, 4H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  119.36, 78.40, 62.46, 37.32, 23.44; IR: 3390, 2956, 2875, 20 1434, 1335, 1204, 1112, 1041, 973. Anal. Calcd for  $\text{C}_9\text{H}_{16}\text{O}_4$ : C, 57.43; H, 8.57. Found: C, 57.48; H, 8.52.

25 **(2R, 3R)-[3-(4-Methoxy-benzyloxymethyl)-1,4-dioxa-spiro[4.4]non-2-yl]-methanol 3b.** To a suspension of  $\text{NaH}$  (175 mg, 4.38 mmol, 1 equiv.) in dry DMF was added alcohol **3a** (824 mg, 4.38 mmol, 1 equiv.) in dry DMF *via* canula. The mixture was cooled to 0 °C and then 4-methoxybenzyl chloride (0.65 ml, 4.82 mmol, 1.1 equiv.) was added dropwise over 20 min. The ice bath was removed and the mixture was stirred at rt for 18 h. Traces of  $\text{NaH}$  30 were decomposed by slow addition of water. The mixture was extracted 3 times with  $\text{CH}_2\text{Cl}_2$ , dried ( $\text{MgSO}_4$ ), and concentrated *in vacuo*. The crude

product was purified on  $\text{SiO}_2$  (hexane:acetone 4:1 containing 10% v/v  $\text{Et}_3\text{N}$ ) to give 879 mg (2.85 mmol, 65%) of compound **3a** as a colorless oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.20-7.30 (m, 2H), 6.80-6.95 (m, 2H), 4.45-4.55 (m, 2H), 3.92-4.00 (m, 1H), 3.82-3.89 (m, 1H), 3.79 (s, 3H), 3.60-3.76 (m, 3H), 3.49 (dd, 1H,  $J_1 = 4.7$ ,  $J_2 = 7.4$ ), 2.45 (bs, 1H), 1.73-1.89 (m, 4H), 1.58-1.73 (m, 4H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  159.29, 129.70, 129.40, 128.52, 119.31, 113.82, 79.56, 76.58, 73.21, 70.14, 62.63, 55.19, 37.26, 37.20, 23.52, 23.40. IR: 3466, 2955, 2872, 1612, 1514, 1248, 1101, 1035. Anal. Calcd for  $\text{C}_{17}\text{H}_{24}\text{O}_5$ : C, 66.21; H, 7.84; Found: C, 66.27, H, 7.76.

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**[2-(Benzylxyloxy-diisopropylamino-phosphoryloxy)-ethyl]-carbamic acid benzyl ester **4**.** To a solution of benzylxybis(*N,N*-diisopropylamino)phosphine (1.538 g, 4.54 mmol, 1.5 equiv.) and 1-*H*-tetrazole (106 mg, 1.51 mmol, 0.5 equiv.) in dry  $\text{CH}_2\text{Cl}_2$ , was added a solution of (2-hydroxyethyl)-carbamic acid benzyl ester (591 mg, 3.03 mmol) in  $\text{CH}_2\text{Cl}_2$ . The mixture was stirred at rt for 3 h, concentrated *in vacuo*, and the crude product was purified on  $\text{SiO}_2$  (hexane:acetone: $\text{Et}_3\text{N}$  6:4:1) to give 886 mg (2.05 mmol, 68%) of compound **4** as an air- and moisture-sensitive colorless oil.  $^1\text{H}$  NMR: (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.20-7.40 (m, 10H), 5.21 (bs, 1H), 5.08 (s, 2H), 4.69 (m, 2H), 3.64-3.79 (m, 2H), 3.56-3.70 (m, 2H), 3.39 (m, 2H), 1.18 (d, 12H,  $J = 5.4$ ).  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ ):  $\delta$  149.1.

**(2-[Benzylxyloxy-[(2*R*, 3*R*)-3-(4-methoxy-benzylxymethyl)-1,4-dioxa-spiro[4.4]non-2-ylmethoxy]-phosphoryloxy]-ethyl]-carbamic acid benzyl ester **5**.** A solution of the monoprotected alcohol **3b** (486 mg, 1.58 mmol, 1 equiv.) and tetrazole (331 mg, 473 mmol, 3 equiv.) in dry  $\text{CH}_2\text{Cl}_2$  (5 mL) was stirred under  $\text{N}_2$  for 5 min at rt. Phosphoramidite **4** (886 mg, 2.05 mmol, 1.3 equiv.) in dry  $\text{CH}_2\text{Cl}_2$  (5 mL) was added *via* canula, and the mixture was stirred for 2 h (until all starting material was consumed). After cooling to -40 °C, *m*CPBA (1.360 g, 6 mmol, 3 equiv., 60%) in  $\text{CH}_2\text{Cl}_2$  (5 mL) was added and the reaction mixture was stirred for 5 min. The cold bath was then removed and stirring was continued for an additional 1 h. The reaction was

diluted with  $\text{CH}_2\text{Cl}_2$  and poured into satd.  $\text{NaHCO}_3$ , and after 20 min of vigorous stirring extracted 3 times with  $\text{CH}_2\text{Cl}_2$ . Combined organic phases were dried ( $\text{MgSO}_4$ ), concentrated *in vacuo*, and the crude product was purified on  $\text{SiO}_2$  (hexane:acetone 3:2) to give 1.033 g (1.57 mmol, 99%) of compound 5 as a colorless oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.28-7.38 (m, 10H), 7.18-7.24 (m, 2H), 6.82-6.88 (m, 2H), 5.44-5.54 (m, 1H), 5.00-5.12 (m, 4H), 4.42-4.51 (m, 2H), 3.99-4.17 (m, 4H), 3.89-3.99 (m, 2H), 3.76 (s, 3H), 3.56 (dd, 1H,  $J_1$  = 3.6,  $J_2$  = 7.5), 3.43-3.50 (m, 1H), 3.33-3.41 (m, 2H), 1.70-1.84 (m, 4H), 1.54-1.70 (m, 4H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  159.30, 156.34, 136.47, 135.65, 135.58, 129.92, 129.79, 129.35, 128.68, 128.63, 128.48, 128.35, 128.07, 128.03, 119.95, 113.80, 77.24, 76.00, 73.18, 69.95, 69.53, 67.46, 66.94, 66.71, 55.21, 53.47, 41.30, 37.26, 37.20, 23.49, 23.42;  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ ):  $\delta$  2.23; IR: 3316, 2956, 1722, 1514, 1250, 1023. Anal. Calcd for  $\text{C}_{34}\text{H}_{42}\text{NO}_{10}\text{P}$ : C, 62.28; H, 6.46; N, 2.14. Found: C, 62.38; H, 6.29; N, 2.18.

(2-{Benzylxy-[(2*R*, 3*R*)-2,3-dihydroxy-4-(4-methoxy-benzylxy)-butoxy]-phosphoryloxy}-ethyl)-carbamic acid benzyl ester 6. To a solution of 5 (2.161 g, 3.3 mmol) in dry THF (100 mL) was added 100 mL of 1 M HCl. The mixture was stirred at rt for 3 h and then satd. aq.  $\text{NaHCO}_3$  was added. The mixture was transferred to a separatory funnel and extracted with  $\text{CH}_2\text{Cl}_2$ . The crude product was purified on  $\text{SiO}_2$  (toluene:acetone 1:4) to give 1.267 g (2.15 mmol, 65%) of the diol 6 as a colorless oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.25-7.35 (m, 10H), 7.17-7.22 (m, 2H), 6.81-6.86 (m, 2H), 5.64-5.75 (m, 1H), 4.95-5.10 (m, 4H), 4.35-4.46 (m, 2H), 3.95-4.12 (m, 4H), 3.78-3.86 (m, 1H), 3.65-3.78 (m, 1H), 3.75 (s, 3H), 3.45-3.55 (m, 2H), 3.30-3.40 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  159.31, 156.51, 136.45, 135.50, 129.77, 129.42, 128.73, 128.64, 128.48, 128.06, 113.83, 73.14, 71.24, 70.50, 69.65, 69.25, 68.90, 66.95, 66.73, 55.22, 41.24;  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ )  $\delta$  0.52; IR: 3349, 2954, 1719, 1612, 1513, 1456, 1248, 1023, 821, 739, 698. Anal. Calcd for  $\text{C}_{29}\text{H}_{36}\text{NO}_{10}\text{P}$ : C, 59.08; H, 6.15; N, 2.38; O, 27.14; P, 5.25. Found: C, 58.96; H, 6.27; N, 2.44; P, 5.44.

**Hexadecanoic acid {(2R, 3R)-3-[benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxy]-2-hexadecanoyloxy-1-(4-methoxybenzyloxymethyl)}-propyl ester 7.** DCC (1.377 g, 6.67 mmol, 3 equiv.), and dimethylaminopyridine (DMAP) (299 mg, 2.45 mmol, 1.1 equiv.) were added 5 in one portion to a solution of **6** (1.311 g, 2.22 mmol, 1 equiv.) and hexadecanoic acid (1.901 g, 6.67 mmol, 3 equiv.) in dry  $\text{CH}_2\text{Cl}_2$ . After stirring for 18 h, the reaction mixture was concentrated *in vacuo* and purified on  $\text{SiO}_2$  (hexane:acetone 4:1) to give 1.68 g (1.58 mmol, 71%) of product **7** as a waxy 10 solid, mp 60 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.26-7.40 (m, 10H), 7.18-7.24 (m, 2H), 6.82-6.88 (m, 2H), 5.08 (s, 2H), 4.95-5.06 (m, 2H), 4.32-4.46 (m, 2H), 3.88-4.24 (m, 4H), 3.76 (s, 3H), 3.43-3.60 (m, 2H), 3.34-3.43 (m, 2H), 2.21-2.32 (m, 4H), 1.50-1.64 (m, 4H), 1.25 (bs, 48H), 0.88 (t, 6H,  $J$  = 5.3);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  172.90, 172.77, 159.35, 129.41, 128.73, 128.65, 128.47, 128.08, 128.01, 113.80, 72.97, 70.04, 69.97, 69.74, 69.66, 69.62, 67.45, 15 67.02, 66.71, 65.65, 55.18, 41.30, 34.15, 31.94, 29.72, 29.67, 29.51, 29.38, 29.31, 29.15, 24.92, 24.86, 22.70, 14.12;  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ )  $\delta$  0.26, 0.14. IR: 2924, 2853, 1741, 1612, 1514, 1456, 1249, 1154, 1112, 1035, 736, 697. Anal. Calcd for  $\text{C}_{61}\text{H}_{96}\text{NO}_{12}\text{P}$ : C, 68.70; H, 9.07; N, 1.31. Found: C, 68.87; H, 8.81; N, 1.32.

20 **Hexadecanoic acid {(2R, 3R)-3-[benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxy]-2-hexadecanoyloxy-1-hydroxymethyl}-propyl ester 8.** To a solution of **7** (234 mg, 0.23 mmol, 1 equiv.) in  $\text{CH}_2\text{Cl}_2$  (23 mL) was added water (0.23 mL) followed by DDQ (103 mg, 0.46 mmol, 2 equiv.). 25 When TLC indicated that the reaction was complete, the mixture was transferred to separatory funnel and washed with 5%  $\text{Na}_2\text{SO}_3$  and satd.  $\text{NaHCO}_3$  (2 x). The aqueous phases were back-extracted once with  $\text{CH}_2\text{Cl}_2$ , and the combined organic phases were dried ( $\text{MgSO}_4$ ), concentrated *in vacuo*, and product was purified on  $\text{SiO}_2$  (hexane:acetone 4:1) to give 125 30 mg (0.13 mmol, 60%) of product **8** as a waxy solid, mp ~60 °C.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.20-7.40 (m, 10H), 5.0-5.16 (m, 6H), 3.92-4.23 (m, 4H), 3.53-3.72 (m, 2H), 3.30-3.45 (m, 2H), 2.80-2.94 (m, 1H), 2.27-2.33 (m, 4H), 1.54-1.64 (m, 4H), 1.25 (bs, 48H), 0.88 (t, 6H,  $J$  = 5.3);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )

δ 173.44, 173.16, 156.46, 136.45, 135.46, 128.81, 128.70, 128.51, 128.13, 128.06, 71.64, 70.05, 69.81, 67.08, 66.80, 65.79, 60.70, 41.30, 34.15, 31.94, 29.73, 29.68, 29.52, 29.38, 29.31, 29.16, 24.90, 22.70, 14.13; <sup>31</sup>P NMR (162 MHz, CDCl<sub>3</sub>) δ 1.68, 1.61; IR: 2917, 2850, 1739, 1467, 1263, 1017. Anal. 5 Calcd for C<sub>53</sub>H<sub>88</sub>NO<sub>11</sub>P: C, 67.27; H, 9.37; N, 1.48. Found: C, 67.32; H, 9.49; N, 1.50.

Hexadecanoic acid {(2R, 3R)-1-[benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxymethyl]-3-(benzyloxy-diisopropylamino-phosphanyloxy)-2-hexadecanoyloxy}-propyl ester **9**. To a solution of benzyltetraisopropylphosphordiamidite (1.164 g, 3.44 mmol, 1.5 equiv.) and tetrazole (80 mg, 1.15 mmol, 0.5 equiv.) in dry CH<sub>2</sub>Cl<sub>2</sub> a solution of ester **8** (2.169 g, 2.30 mmol, 1 equiv.) in CH<sub>2</sub>Cl<sub>2</sub> was added *via* canula. After 2 h, the reaction was concentrated *in vacuo* and the residue was purified on SiO<sub>2</sub> (ethyl acetate:toluene 4:1 containing 5% Et<sub>3</sub>N) to give 2.190 g (1.85 mmol, 81%) of phosphoramidite **9**. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.20-7.40 (m, 10H), 5.0-5.16 (m, 6H), 3.92-4.23 (m, 4H), 3.53-3.72 (m, 2H), 3.30-3.45 (m, 2H), 2.80-2.94 (m, 1H), 2.27-2.33 (m, 4H), 1.54-1.64 (m, 4H), 1.25 (bs, 48H), 0.88 (t, 6H, J=5.3). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 172.91, 172.75, 139.18, 135.58, 20 135.52, 128.74, 128.67, 128.49, 128.31, 128.26, 128.09, 128.02, 127.36, 127.29, 126.96, 126.93, 70.59, 69.72, 67.01, 66.72, 65.70, 65.37, 65.20, 61.69, 43.10, 34.18, 31.95, 29.73, 29.68, 29.52, 29.38, 29.33, 29.17, 24.88, 24.58, 22.70, 14.13; <sup>31</sup>P NMR (162 MHz, CDCl<sub>3</sub>) δ 150.18, 149.79, 0.32, 0.22. 25 Hexadecanoic acid 1-[benzyloxy-(2-benzyloxycarbonylamino-ethoxy)-phosphoryloxymethyl]-2-hexadecanoyloxy-3-[1-benzyloxy-phosphoryloxy-3-benzyloxy-2,6-bis(benzyloxymethoxy)-4,5-bis-(bis-benzyloxy-phosphoryloxy)-D-myo-inositol]-propyl ester **10**. To a solution of 4,5-head group (4,5-HG, 250 mg, 0.24 mmol, 1 equiv.) and tetrazole (51 mg, 0.73 mmol, 3 equiv.) in dry CH<sub>2</sub>Cl<sub>2</sub> was added a solution of phosphoramidite **9** (345 mg, 0.29 mmol, 1.2 equiv.) in CH<sub>2</sub>Cl<sub>2</sub>. The mixture was stirred at rt for about 3 h, cooled to -40 °C and *m*CPBA (210 mg, 0.73 mmol, 3 equiv., 60%) was added. After 15 min, the cold bath was removed 30

and stirring was continued for additional 1 h. The reaction mixture was diluted with  $\text{CH}_2\text{Cl}_2$  and poured into a solution of 5%  $\text{Na}_2\text{SO}_3$  and satd.  $\text{NaHCO}_3$ . The mixture was extracted 3 times with  $\text{CH}_2\text{Cl}_2$  and combined organic fractions were dried over  $\text{MgSO}_4$ , concentrated *in vacuo*, and purified on  $\text{SiO}_2$  (hexane:acetone 3:2) to give 306 mg (0.14 mmol, 59%) of product **10** as a colorless oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.00-7.35 (m, 50H), 5.55-5.95 (m, 1H), 5.15-5.35 (m, 2H), 4.40-5.15 (m, 27H), 4.20-4.40 (m, 2H), 3.90-4.20 (m, 6H), 3.45-3.60 (m, 1H), 3.25-3.45 (m, 2H), 2.10-2.30 (m, 4H), 1.40-1.60 (m, 4H), 1.05-1.35 (m, 48H), 0.88 (t, 6H,  $J$  = 5.3);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  172.59, 138.09, 137.71, 137.25, 136.87, 136.56, 136.23, 136.17, 136.10, 136.06, 135.99, 135.90, 135.82, 135.51, 135.54, 128.65, 128.44, 128.34, 128.26, 128.02, 127.84, 127.75, 127.42, 96.64, 95.46, 78.99, 77.61, 77.34, 76.93, 76.60, 74.89, 72.79, 72.02, 70.45, 69.90, 69.70, 69.44, 69.12, 66.97, 66.62, 65.20, 41.29, 33.97, 31.92, 29.70, 29.67, 29.66, 29.51, 29.35, 29.29, 29.13, 24.78, 22.68, 14.12;  $^{31}\text{P}$  NMR (162 MHz,  $\text{CDCl}_3$ )  $\delta$  0.35, -0.15, -0.56 (ratio 1:2:1). IR: 2924, 2853, 1743, 1455, 1273, 1022. Anal. Calcd for  $\text{C}_{117}\text{H}_{153}\text{NO}_{27}\text{P}_4$ : C, 65.99; H, 7.24; N, 0.66; P, 5.82. Found: C, 65.75; H, 7.24; N, 0.73; P, 6.12.

**20 1-[(2R, 3R)-4-(2-Aminoethoxyphosphoryloxy)-2,3-di-O-palmitoylbutoxyphosphoryloxy]-4,5-myo-bisphosphate **11**.** To a solution of compound **10** (193.4 mg, 0.091 mmol) in a mixture of THF/water (4:1, v/v, 50 mL) was added 10% palladium on charcoal (387 mg). The mixture was shaken for 18 h at rt under 60 psi of  $\text{H}_2$ . The catalyst was removed by filtration and solvent was removed *in vacuo*. The crude product was redissolved in water and stirred for 3 h with Dowex 50X-100 resin ( $\text{Na}^+$  form). The resin was removed by filtration and the filtrate was lyophilized to give 77.3 mg (0.062 mmol, 62%) as the sodium salt. The dried crude product was used for coupling with activated (N-hydroxysuccinimidyl, or "NHS") esters as described below.  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  5.15-5.30 (m, 2H), 4.10-4.30 (m, 2H), 3.85-4.10 (m, 7H), 3.75-3.85 (m, 1H), 3.6-3.7 (m, 1H), 3.4-3.5 (m, 1H), 3.18 (bs, 2H), 2.1-2.5 (m, 4H), 1.4-1.6 (bs, 4H), 1.18 (bs, 48H), 0.76 (bs, 6H).  $^{31}\text{P}$  NMR (162 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  2.36, 1.80, 1.09, 0.56 (ratio 1:1:1:1), MS MALDI (free acid):

1146 (M+Na), 950 (M+3Na-C<sub>15</sub>H<sub>31</sub>CO), 928 (M+2Na-C<sub>15</sub>H<sub>31</sub>CO), 906 (M+Na-C<sub>15</sub>H<sub>31</sub>CO), 884 (M-C<sub>15</sub>H<sub>31</sub>CO).

**General procedure for coupling with NHS esters.** To a solution of compound 11 (~10  $\mu$ mol, 1 equiv.) in 0.5 M TEAB (0.5 mL, pH 7.5) was added a solution of appropriate NHS ester (~12  $\mu$ mol, 1.2 equiv.) (three of which were obtained from Molecular Probes, Inc.) in 0.5 mL of dimethylformamide (DMF) was added. PROXYL-SE was prepared as described in Rauch, M.; Ferguson, C.; Prestwich, G. D.; Cafiso, D. *J. Biol. Chem.* 2002. The mixture was stirred at rt for 18 h, and solvents were then removed *in vacuo*. The residue was washed 4 times with acetone and then purified on DEAE-cellulose column with a step gradient of TEAB (0 to 2 M). The desired fractions were pooled, lyophilized, converted by ion exchange into a sodium salt, and lyophilized again.

**15 Biotin derivative 12a.** Reaction of 11 (9.4 mg, 7.5 mmol) with Biotin-X, SE (4.4 mg, 9.7 mmol) yielded 6.4 mg (4 mmol, 53%) of 12a. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O)  $\delta$  5.20-5.40 (m, 4H), 4.50-4.60 (m, 1H), 4.30-4.45 (m, 1H), 3.70-4.45 (m, 9H), 3.60-3.70 (m, 1H), 3.30-3.45 (m, 1H), 3.05-3.20 (m, 4H), 2.65-2.95 (m, 2H), 2.00-2.50 (m, 8H), 1.40-1.80 (m, 12H), 0.90-1.40 (m, 52H), 0.70-0.90 (m, 6H); <sup>31</sup>P NMR (162 MHz, D<sub>2</sub>O)  $\delta$  3.22, 2.32, 1.34, 0.41 (ratio 1:1:1:1). MS MALDI (free acid): 1528 (M+3Na), 1506 (M+2Na), 1484 (M+Na), 1462 (M-H), 1223 (M-H-C<sub>15</sub>H<sub>31</sub>CO), 1122 (M-H-biotin). HR MALDI: C<sub>60</sub>H<sub>113</sub>N<sub>4</sub>O<sub>26</sub>P<sub>4</sub>S [M-H]<sup>-</sup> calcd: 1461.60388, found: 1461.60491.

**25 NBD Derivative 12b.** Reaction of 11 (9.9 mg, 7.9 mmol) with NBD-X, SE (4.0 mg, 10.2 mmol) afforded 7.7 mg (5 mmol, 64%) of 12b. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O):  $\delta$  8.10-8.30 (m, 1H), 6.00-6.20 (m, 1H), 5.10-5.30 (m, 2H), 3.65-4.20 (m, 12H), 3.20-3.65 (m, 5H), 2.95-3.25 (m, 4H), 2.20-2.40 (m, 2H), 2.15-2.25 (m, 4H), 1.65-1.80 (m, 2H), 1.50-1.65 (m, 2H), 1.30-1.50 (m, 4H), 0.80-1.30 (m, 50H), 0.50-0.80 (m, 6H); <sup>31</sup>P NMR (162 MHz, D<sub>2</sub>O):  $\delta$  4.03, 2.92, 1.37, 0.47 (ratio 1:1:1:1). MS MALDI (free acid): 1161 (M-C<sub>15</sub>H<sub>31</sub>CO).

Fluorescein derivative **12c**. Reaction of **11** (9.5 mg, 7.6 mmol) with 6-FAM-SE (4.7 mg, 9.8 mmol) yielded 9.1 mg (5.6 mmol, 74%) of **12c**. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ 7.30-7.50 (m, 8H), 5.20-5.40 (m, 2H), 3.10-4.30 (m, 14H), 2.20-2.40 (m, 4H), 1.40-1.60 (m, 4H), 0.90-1.40 (m, 48H), 0.60-0.90 (m, 6H); <sup>31</sup>P NMR (162 MHz, D<sub>2</sub>O): δ 4.94, 4.33, 1.21, 0.65 (ratio 1:1:1:1). MS MALDI (free acid): 1243 (M-C<sub>15</sub>H<sub>31</sub>CO), 1123 (M-fluorescein), 884 (M-C<sub>15</sub>H<sub>31</sub>CO-fluorescein), 841 (M-C<sub>15</sub>H<sub>31</sub>CO-fluorescein-aminoethyl).

PROXYL derivative **12d**. Reaction of **11** (9.8 mg, 7.8 mmol) with PROXYL-SE (2.9 mg, 10.1 mmol) afforded 6.1 mg (4.3 mmol, 55%) of **12d**. MS MALDI (free acid): 1292 (M'), 1123 (M-proxyl), 1053 (M-C<sub>15</sub>H<sub>31</sub>CO), 884 (M-C<sub>15</sub>H<sub>31</sub>CO-proxyl). HR MALDI: C<sub>53</sub>H<sub>103</sub>N<sub>2</sub>O<sub>25</sub>P<sub>4</sub> [M]<sup>+</sup> calcd: 1291.57950; found: 1291.57679.

The resulting exemplary Pea-PIP<sub>n</sub>, 2,3-diacylthreitol-based Pea-PtdIns(4,5)P<sub>2</sub>, ("Pea-PIP2") possesses a phosphatidylethanolamine (Pea) head group at the 1-position and a phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) head group at the 4-position. Reporters (biotin, fluorophores, spin label) were covalently attached to the free amino group of the Pea, such that these reporters were targeted to the lipid-water interface. See Figure 5. The diacyl moieties allow incorporation of Pea-PIP<sub>2</sub> into a lipid bilayer, while the PtdIns(4,5)P<sub>2</sub> moiety in the aqueous layer is specifically recognized by PtdIns(4,5)P<sub>2</sub>-specific binding proteins. Reaction of the free Pea amino group of with four N-hydroxysuccinimidyl (NHS) esters afforded the corresponding biotinylated derivative **C-2a**, the fluorescent N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) and 6-carboxyfluorescein derivatives **C-2b** and **C-2c**, and the spin-labeled 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXYL) derivative **C-2d**. Preliminary biological results are described below for exemplary biotinylated and fluorescent derivatives.

Example 2. Synthesis of Pea-PIP<sub>n</sub>s Having Eight Different Naturally  
Occurring Phosphoinositide Head Groups:

Synthetic representatives of eight naturally occurring phosphoinositide head groups have been incorporated into Pea-PIP<sub>n</sub>s of the present invention. 5 The synthetic method for producing these was as described above for Pea-PI(4,5)P<sub>2</sub> and its reporter derivatives. The synthetic strategy for producing all eight of these Pea-PIP<sub>n</sub>s is described in Table 1 and Figure 2. The head groups used in this Example to produce Pea-PIP<sub>n</sub>s of the invention are, PI, PI(3)p, PI(4)P, PI(5)P, PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>.

10

TABLE 1.

Headgroup	Protected	Deprotected
PI,	R <sub>1</sub> ,R <sub>1</sub> ,R <sub>3</sub> =Bn	R <sup>4</sup> , R <sup>5</sup> , R <sup>6</sup> =H
PI(3)p	R <sub>1</sub> ,R <sub>2</sub> =Bn, R <sub>3</sub> =PO <sub>3</sub> Bn <sub>2</sub>	R <sup>1</sup> ,R <sup>2</sup> =H, R <sup>3</sup> =PO <sub>3</sub> H <sub>2</sub>
PI(4)P	R <sub>1</sub> , R <sub>3</sub> =Bn, R <sub>2</sub> =PO <sub>3</sub> Bn <sub>2</sub>	R <sup>1</sup> , R <sup>3</sup> =H, R <sup>2</sup> =PO <sub>3</sub> H <sub>2</sub>
PI(5)P	R <sub>2</sub> , R <sub>3</sub> =Bn, R <sub>1</sub> =PO <sub>3</sub> Bn <sub>2</sub>	R <sup>2</sup> , R <sup>3</sup> =H, R <sup>1</sup> =PO <sub>3</sub> H <sub>2</sub>
PI(3,4)P <sub>2</sub>	R <sub>1</sub> =Bn, R <sub>2</sub> , R <sub>3</sub> =PO <sub>3</sub> Bn <sub>2</sub>	R <sup>1</sup> =H, R <sup>3</sup> =PO <sub>3</sub> H <sub>2</sub>
PI(3,5)P <sub>2</sub>	R <sub>2</sub> =Bn, R <sub>1</sub> , R <sub>3</sub> =PO <sub>3</sub> Bn <sub>2</sub>	R <sup>2</sup> =H, R <sup>1</sup> , R <sup>3</sup> =PO <sub>3</sub> H <sub>2</sub>
PI(4,5)P <sub>2</sub>	R <sub>3</sub> =Bn, R <sub>1</sub> , R <sub>2</sub> =PO <sub>3</sub> Bn <sub>2</sub>	R <sup>3</sup> =H, R <sup>1</sup> , R <sup>2</sup> =PO <sub>3</sub> H <sub>2</sub>
PI(3,4,5)P <sub>3</sub>	R <sub>1</sub> , R <sub>2</sub> , R <sub>3</sub> =PO <sub>3</sub> Bn <sub>2</sub>	R <sup>1</sup> , R <sup>2</sup> , R <sup>3</sup> =PO <sub>3</sub> H <sub>2</sub>

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Example 3. Synthesis of Linker-Modified Derivatives of Pea-PIP<sub>n</sub>s:

a. Hydrophilic linker-modified Pea-PIP<sub>n</sub> analogs. Preliminary data from immobilization of Pea-PIP<sub>n</sub>s and a hydrophilic linker-modified analog to functionalized surfaces suggests that increasing the distance between the 20 Pea-PIP<sub>n</sub> head group and the probe moiety may increase ligand recognition. A hydrophilic linker-modified Pea-PIP<sub>n</sub> derivative was synthesized in order to increase the distance between the Pea-PIP<sub>n</sub> head group and the probe moiety (see Figure 6). In a first example of linker extension, amino-PEG-amide linker-extended Pea-PI(4,5)P<sub>2</sub> was prepared from the parent Pea-PI(4,5)P<sub>2</sub> by 25 coupling the primary amine with the NHS ester of a 16-atom linker purchased as a Fmoc protected activated ester (available commercially, for example from Quanta Biodesign, Inc.). This derivative was examined for binding to the

PLC  $\delta$ 1 PH domain using AlphaScreen<sup>®</sup> (available from PerkinElmer Life Sciences), PIP Arrays<sup>™</sup> (available from Echelon Biosciences, Inc.), and immobilized on NHS-activated plates or beads. Increased binding to a pleckstrin homology domain was observed with the addition of the hydrophilic

5 linker.

b. Additional PEG linker Pea-PIP<sub>n</sub> analogs. Various lengths of poly(ethylene glycol)-based linkers can be used for preparation of alternative linker-modified Pea-PIP<sub>n</sub>s. In a particular embodiment, PEG linkers were prepared from commercially available di-, tri-, tetra-, and/or penta(ethylene glycols). In the first step, the linkers were transformed into mono *p*-toluenesulfonates (Bauer, H., Stier, F., Petry, C., Knorr, A., Stadler, C., and Staab, H. A. (2001) *European Journal of Organic Chemistry*, 3255-3278), then converted into the oligo-PEG- $\omega$ -aminoalcohols (Nelissen, H. F. M., Venema, F., Uittenbogaard, R. M., Feiters, M. C., and Nolte, R. J. M. (1997) *Journal of the Chemical Society, Perkin Transactions 2: Physical Organic Chemistry*, 2045-2053; Bramson, H. N., Corona, J., Davis, S. T., Dickerson, S. H., Edelstein, M., Frye, S. V., Gampe, R. T., Jr., Harris, P. A., Hassell, A., Holmes, W. D., Hunter, R. N., Lackey, K. E., Lovejoy, B., Luzzio, M. J., Montana, V., Rocque, W. J., Rusnak, D., Shewchuk, L., Veal, J. M., Walker, D. H., and Kuyper, L. F. (2001) *Journal of Medicinal Chemistry* 44, 4339-4358), and finally protected with CbzCl in dichloromethane/triethylamine (Roy, B. C., and Mallik, S. (1999) *Journal of Organic Chemistry* 64, 2969-2974). In this Example, linker extensions including 2-(2-Amino-ethoxy)-ethanol and 2-(2-[2-(2-Amino-ethoxy)-ethoxy]-ethoxy)-ethanol were introduced at an early stage of Pea-PI(4,5)P<sub>2</sub> synthesis, specifically during the preparation of the phosphoramidites. Figure 7 illustrates the synthetic strategy for preparation of four oligo-PEG linkers. Relatively short PEG linkers are chosen to avoid floppiness, foldback, and heterogeneity often experienced with MW 700 or 1500 or 3400 PEG derivatives. In certain embodiments of the present invention, PEG linkers can be of any size. In particular embodiments, such PEG linkers may range from MW 88-3400 (44-20,000 Daltons). Suitably protected aminoalcohols are next converted into the corresponding phosphoramidites and coupled with specifically protected threitol as shown in

Figure 8. Following coupling, oxidation, and deprotection as previously described and shown in Figure 2 and Table 1, three oligoethylene glycol phosphoramidite modified Pea-PIP<sub>n</sub> analogs were obtained.

5 **Example 4. Pea-PIP<sub>n</sub>s are Substrates for Lipid Phosphatase or Kinase:**

This Example demonstrates further that Pea-PIP<sub>n</sub>s of the present invention are effective substrates for lipid phosphatases and kinases. In this Example, Pea-PIP<sub>n</sub>s were shown to be effective and specific substrates for recombinantly expressed and purified recombinant human PTEN/MMAC1 (acronyms for Phosphatase and Tensin homolog and mutated in multiple advanced cancers). PTEN/MMAC1 is a lipid phosphatase that removes the 3' phosphate from PI(3,4,5)P<sub>3</sub> to produce PI(4,5)P<sub>2</sub>. According to this Example, purified Glutathione S-Transferase (GST)-tagged, PTEN (0.24 mg/ml in 50% glycerol 50% elution buffer) was added to 125 pmol Pea-PI(3,4,5)P<sub>3</sub> in 50 ul reaction buffer (100 mM TRIS, pH 8.0, 10 mM DTT) (chemicals from Sigma-Aldrich Life Science, St. Louis, MO). The reactions were incubated for one hour at 37 °C, and detected using an AlphaScreen™ assay. A standard curve of the product was generated by adding serial dilutions of Pea-PI(4,5)P<sub>2</sub> as the competitor to separate wells in the same plate. Results of this assay showed that two separate enzyme preparations of PTEN were active against Pea-PI(3,4,5)P<sub>3</sub>. Using the standard curve, it was estimated that 4-8% of the Pea-PI(3,4,5)P<sub>3</sub> substrate was converted to Pea-PI(4,5)P<sub>2</sub> during the course of the reaction. These results show that members of this unique and novel class of phosphoinositide analogs are acted on as substrates in assay platforms for lipid kinases and phosphatases. Although this Example uses PTEN and detection of Pea-PI(4,5)P<sub>2</sub>; however, it is envisioned that this system extends to any combination of Pea-PIP<sub>n</sub> headgroup detection and any phosphoinositide kinase or phosphatase.

**Example 5. Immobilized Pea-PIP<sub>n</sub>s are substrates for Lipid Recognizing Proteins.**

In this Example, immobilized Pea-PIP<sub>n</sub>s are readily and specifically recognized by lipid recognition proteins. According to this Example, six different quantities (from 200 pmol to 6 pmol) of PtdIns(3,4)P<sub>2</sub>, PtdIns(3,5)P<sub>2</sub>, PtdIns(4,5)P<sub>2</sub>, Pea-PI(4,5)P<sub>2</sub>, PtdIns(3,4,5)P<sub>3</sub>; Pea-PI(3,4,5)P<sub>3</sub> PI and PE were spotted onto nitrocellulose, and binding of Glutathione S-Transferase (GST)-PLC δ<sub>1</sub>-PH and GST-General Receptor for Phosphoinositides (Grp1)-PH constructs was examined by a protein-overlay technique (described in Dowler, S., Kular, G., and Alessi, D. R. (2002) *Sci STKE* 2002, PL6) (recombinant GST tagged PLC δ<sub>1</sub>- PH and Grp1-PH domain proteins used in this Example were expressed in *E. coli* then purified using glutathione affinity resin (Amersham Biosciences, Piscataway, NJ). The results of this binding assay show that neither lipid-recognizing protein (LRP) recognizes the PE or PI control lipids, nor do they recognize the non-cognate phosphoinositides, but both LRP<sub>s</sub> showed dose-dependent recognition of the correct immobilized phosphoinositide. Significantly, both Pea-PI(4,5)P<sub>2</sub> and Pea-PI(3,4,5)P<sub>3</sub> are able to bind the correct protein at lower concentrations than the corresponding diC<sub>16</sub> phosphoinositides, demonstrating that the Pea-PIP<sub>n</sub>s have improved LRP binding capabilities compared to the current standard PIP<sub>n</sub> lipids.

**Example 6. Biotinylated Pea-PIP<sub>n</sub>s are Substrates for Lipid Recognizing Proteins.**

This example demonstrates that a phosphoinositide head-group specific lipid-recognizing protein (LRP), namely GST-Phospholipase Cδ<sub>1</sub> (PLC δ<sub>1</sub>) PH domain, will easily bind to the biotinylated derivative of Pea-PI(4,5)P<sub>2</sub>, even when bound to a surface.

According to this example, biotinylated lipid Pea-PI(4,5)P<sub>2</sub> was bound to a streptavidin-coated donor bead and the GST-tagged PLC δ<sub>1</sub>-PH domain was attached to an anti-GST-coated acceptor bead. A luminescent signal quantitatively reported the interaction between the biotinylated lipid and the binding protein. Further, in the absence of a lipid or a specific binding protein,

no signal was seen. In the presence of 0.1 pmol/well of specific binding protein, biotinylated Pea-PI(4,5)P<sub>2</sub> showed a dose-dependent increase in luminescent signal up to 1 pmol per well.

In this experiment, a bioluminescence assay (AlphaScreen™, Perkin-  
5 Elmer Life Sciences, Boston, MA) was used to establish biochemical relevance of the claimed compounds. Binding of Pea-PI(4,5)P<sub>2</sub> to PLC δ<sub>1</sub> was determined using recombinant GST tagged PLC δ-PH domain protein that was expressed in *E. coli*, then purified using glutathione affinity resin (Amersham Biosciences, Piscataway, NJ). Several concentrations of purified  
10 protein and biotinylated-Pea-PIP<sub>2</sub> (all phosphoinositides were from Echelon Biosciences Inc, SLC, UT) were combined in a white 384-well microplate (Optiplate™, Packard Bioscience, Meriden, CT). Streptavidin donor and Anti-GST acceptor beads (Perkin-Elmer life sciences) were then added in a light protected area, so that the final bead concentration is 5 µg/mL in 25 µL  
15 final reaction volume (all dilutions in AlphaScreen assay buffer, Tris-Buffered Saline pH 7.5, 0.1% Tween-20, 0.1% Bovine Serum Albumin). The plate was protected from light and incubated for 2 hours at room temperature before reading with the AlphaScreen mode of a Fusion instrument (Perkin-Elmer life sciences).

20 In addition, competitive binding assays were conducted in which the GST-PLC δ<sub>1</sub>-PH protein was pre-incubated for 30 min with 10 nM to 10 fM of unlabeled di-C<sub>4</sub> PtdIns(4,5)P<sub>2</sub>, PtdIns(3,4,5)P<sub>3</sub>, or Pea-PI(4,5)P<sub>2</sub> prior to addition of the other reagents, using biotinylated Pea-PI(4,5)P<sub>2</sub> as the probe lipid. Over 100-fold selectivity was observed for displacement of the  
25 GST-PLC δ<sub>1</sub>-PH from binding to Pea-PI(4,5)P<sub>2</sub> by the di-C<sub>4</sub> PtdIns(4,5)P<sub>2</sub>, relative to di-C<sub>4</sub> PtdIns(3,4,5)P<sub>3</sub>. A further increase in binding to the PH domain of GST-PLC δ<sub>1</sub> was observed using the non-biotinylated version of Pea-PI(4,5)P<sub>2</sub>.

30 **Example 7. Specific Recognition and Binding by Anti-PIP<sub>n</sub> Antibody to Pea-PIP<sub>n</sub>s Covalently Bound to a Surface:**

The ability of lipid recognition proteins to specifically recognize and bind to Pea-PIP<sub>n</sub>s was tested by coupling Pea-PIP<sub>n</sub>s (as well as amino PIPs

with the same headgroups as controls) to polystyrene microtiter plates. Briefly, 50 pmol per well of amino PI(4,5)P<sub>2</sub> (PIP2), amino PI(3,4,5)P<sub>3</sub> (PIP3), Pea-PI(4,5)P<sub>2</sub> (Pea-PIP2), and Pea-PI(3,4,5)P<sub>3</sub> (Pea-PIP3) lipids in 100 µL PBS were coupled via their primary amine functional groups to triplicate wells of a Maleic-Anhydride activated 96-well plate (Pierce, Rockford, Illinois). Underivatized groups were reacted with 200 µL Tris-Glycine-SDS overnight before blocking with 200 µL 0.02 % Ovalbumin in TBS. The plate was then incubated with anti-PIP<sub>3</sub> antibody (for example, NN111.1.1, MBL International, Watertown, MA) for one hour at room temperature on an orbital shaker. The plate was washed 3-5 times with 200 µL/well of TBS containing 0.1% Tween-20. Specific binding was subsequently visualized by incubating with anti-mouse-HRP (horseradish peroxidase) secondary antibody (Sigma, St. Louis, MO), washing as before, then adding tetramethylbenzidine (TMB) developing reagent (Sigma) and reading the absorbance at 450 nm in a plate reader.

15 Anti-PIP3 antibody correctly and specifically bound to Pea-PI(3,4,5)P<sub>3</sub>, demonstrating that, similar to the control lipids, Pea-PIP<sub>3</sub> (but not Pea-PIP<sub>2</sub>) was recognized by the specific antibody. In addition, Pea-PIP<sub>3</sub> gave an increased signal compared to  $\omega$  - amino alkanoyl PIP<sub>3</sub>. These results show that immobilizing Pea-PIPs either by non-specific adsorption or covalent coupling allows the structural features necessary for recognition by antibodies and lipid recognizing proteins (namely, the headgroup and two fatty-acyl chains) to be better positioned than in traditional PIPs.

20

**Example 8. Intracellular uptake of Pea-PIPs:**

25 Pea- PIP<sub>n</sub>s can be delivered into living cells using a commercially available system (Echelon Bioscience Shuttle PIP™ system (Salt Lake City, Utah)). A fluorescent Pea-PI(4,5)P<sub>2</sub> analog was delivered to cells using the Shuttle PIP™ technology. This method has previously been used to deliver fluorescent PtdInsP<sub>n</sub> analogs, PtdIns(3,4)P<sub>2</sub> for Protein Kinase B (Akt) activation, and PtdIns(3,4,5)P<sub>3</sub> to induce cell migration. 3T3-L1 preadipocyte cells were seeded onto an 8-well cover-glass chamber slide in complete media. After 24 hrs the cells were approximately 60% confluent and the media was replaced with 100 µL serum free media for 45 minutes before

30

adding a mixture of fluorescent Pea-PI(4,5)P<sub>2</sub>-NBD and Histone H1 carrier (premixed and incubated at room temperature for 10 minutes, final concentration of Pea-PI(4,5)P<sub>2</sub>-NBD was 12.5  $\mu$ M; and Histone carrier, 2.5  $\mu$ M). After 30 minutes, the cells were imaged with a Bio-Rad confocal microscope at 300x magnification. Results of this assay showed that Pea-PI(4,5)P<sub>2</sub>-NBD localized to intracellular compartments with bright staining associated in specific regions of the plasma membrane. This pattern of intracellular localization positions Pea-PI(4,5)P<sub>2</sub> correctly in the cell to substitute for endogenous PtdIns(4,5)P<sub>2</sub> in signaling pathways and cell-based assays.

**Example 9. PeaPIPns can substitute for synthetic PIPns on PIP Strip\***

**Products:**

Pea-PIPns can substitute for synthetic PIPns on PIP Strip\* products (Echelon, Salt Lake City, Utah). Briefly, 100, 50, 25, 12.5, 6.25, and 3.125 pmol of lipids in organic solvent were spotted onto PVDF membrane (Amersham, Boston, MA) and allowed to dry before blocking the membrane with 0.1 % Ovalbumin in TBS. The membranes were then incubated with GST-PH domain proteins (LRPs) specific for PI(4,5)P<sub>2</sub> or PI(3,4,5)P<sub>3</sub> for one hour at room temperature on an orbital shaker. Binding was visualized by subsequent incubations of anti-GST and anti-Mouse-HRP secondary antibodies followed by ECL (enhanced chemiluminescence) detection and exposure to photographic film. The Pea-PIPn's demonstrated the correct specificity for LRP binding and were superior to regular synthetic PIPs by immobilizing both PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>-specific proteins at lower lipid concentrations.

**Example 10. Pea-PIPs are capable of Adhesion to Microtiter Plates:**

Binding specificity of Pea-PIPs was further tested by coupling Pea-PIP2 and Pea-PIP3 (as well as amino PIPs with the same headgroups as controls) to polystyrene microtiter plates. Briefly, 50 pmol per well of amino PI(4,5)P<sub>2</sub> (PIP<sub>2</sub>), amino PI(3,4,5)P<sub>3</sub> (PIP<sub>3</sub>), Pea-PI(4,5)P<sub>2</sub> (PEA-PIP<sub>2</sub>), and Pea-PI(3,4,5)P<sub>3</sub> (PEA-PIP<sub>3</sub>) lipids in 100  $\mu$ L PBS were coupled via their

primary amine functional groups to triplicate wells of a Maleic-Anhydride activated 96-well plate (Pierce, Rockford, Illinois). Underivatized groups were reacted with 200  $\mu$ L Tris-Glycine-SDS overnight before blocking with 200  $\mu$ L 0.02 % Ovalbumin in TBS. The plate was then incubated with anti-PIP<sub>3</sub> 5 antibody (for example, NN111.1.1, MBL International, Watertown, MA) for one hour at room temperature on an orbital shaker. The plate was washed 3-5 times with 200  $\mu$ L/well of TBS containing 0.1% Tween-20. Specific binding was subsequently visualized by incubating with anti-mouse-HRP secondary antibody (Sigma, St. Louis, MO), washing as before, then adding 10 Tetramethylbenzadine (TMB) developing reagent (Sigma) and reading the absorbance at 450 nm in a plate reader. Similar to the control lipids; Pea-PIP<sub>3</sub> (but not Pea-PIP<sub>2</sub>) was recognized by the specific antibody. In addition Pea-PIP<sub>3</sub> gave an increased signal compared to amino PIP<sub>3</sub>, similar to the nitrocellulose experiment. This Example demonstrates that immobilization of 15 Pea-PIPs either by non-specific adsorption or covalent coupling allows the structural features necessary for recognition by antibodies and lipid recognizing proteins (namely, the headgroup and two fatty-acyl chains) to be better positioned than traditional PIPs.

20 **Example 11. Pea-PIPs are capable of transfer into living cells:**

Pea-PIPns can be delivered into living cells using Echelon's Shuttle PIP system (Salt Lake City, Utah). 3T3-L1 fibroblasts in modified DMEM media (Gibco BRL, Maryland) were seeded onto an 8-well coverglass chamber slide ((Nalge Nunc International, Naperville, IL), 200  $\mu$ L per chamber 25 in complete media. After 24 hrs the cells were 60% confluent and the media was replaced with 100  $\mu$ L serum free media for 45 minutes before 2.5  $\mu$ L of 5 mM fluorescent Pea-PI(4,5)P<sub>2</sub>-NBD was added to 6.25  $\mu$ L of 200  $\mu$ M Histone H1 (Sigma, St. Louis, MO) and incubated at room temperature for 10 minutes. Then 16.25  $\mu$ L serum-free media was added to the PIP/Histone 30 complex and incubated for an additional 5 minutes before it was added to the cells in a final volume of 125  $\mu$ L and a final concentration of 100  $\mu$ M Pea-PI(4,5)P<sub>2</sub> and 10  $\mu$ M Histone H1. The cells on coverslips were imaged with a Bio-Rad confocal microscope at 60x magnification. This procedure was

repeated in a separate experiment with a final Pea-PI(4,5)P<sub>2</sub> concentration of 12.5  $\mu$ M and Histone concentration of 2.5  $\mu$ M then visualized at 300x magnification. Pea-PI(4,5)P<sub>2</sub>-NBD clearly localized to intracellular compartments with bright staining associated in several regions of the plasma membrane. This pattern of intracellular localization positioned Pea-PI(4,5)P<sub>2</sub> correctly in the cell to substitute for endogenous PI(4,5)P<sub>2</sub> in signaling pathways and cell-based assays.

5

**Example 12.: Pea-PIPn's can substitute for synthetic PIPs in lipid kinase and lipid phosphatase assay development programs**

10 A Phosphoinositide head-group specific lipid-recognizing protein (LRP), namely GST-Phospholipase Cd1 PH domain, will easily bind to the biotinylated derivative of Pea PI(4,5)P<sub>2</sub>. An AlphaScreen technology (Perkin-Elmer Life Sciences, Boston, MA) is utilized for our lipid-protein binding assay 15 in this example.

15 AlphaScreen (for Amplified Luminescent Proximity Homogenous Assay) is a chemiluminescent, bead-based assay performed in white micro-titer plates. When excited by 680 nm laser light, donor beads convert ambient oxygen to a more excited singlet state. When an acceptor bead is in close 20 proximity to the donor bead (through a biological interaction) singlet oxygen reacts with a thioxene derivative in the acceptor bead generating chemiluminescence light of 370 nm wavelength which further excites fluorophores on the same acceptor bead emitting light at 520-620 nm.

25 Binding of PEA PI(4,5)P<sub>2</sub> to PLCd1 was determined by expressing recombinant GST tagged PLC-d1- PH domain protein in E. coli which was purified using glutathione affinity resin (Amersham Biosciences, Piscataway, NJ). Several concentrations of purified protein and biotinylated-PEA PIP<sub>2</sub> (all phosphoinositides were from Echelon Biosciences Inc, SLC, UT) were combined in a white 384-well microplate (OptiplateTM, Packard Bioscience, 30 Meriden, CT). Streptavidin donor and Anti-GST acceptor beads (Perkin-Elmer life sciences) were then added in a light protected area, so that the final bead concentration is 5  $\mu$ g/mL in 25  $\mu$ L final reaction volume (all dilutions in AlphaScreen assay buffer, Tris-Buffered Saline pH 7.5, 0.1% Tween-20, 0.1%

Bovine Serum Albumin). The plate was protected from light and incubated for 2 hours at room temperature before reading with the AlphaScreen mode of a Fusion instrument (Perkin-Elmer life sciences).

A strong binding interaction between biotinylated Pea-PI(4,5)P<sub>2</sub> and the 5 PH domain of PLC- $\delta$ -1 was observed with maximum signal at 0.8 pmol/well biotinylated Pea-PI(4,5)P<sub>2</sub> and 0.4 pmol/well PLC-  $\delta$  -1. Half these concentrations also produced a strong signal and were used as binding partners in the next series of experiments to demonstrate specificity of Pea-PIP binding to PLC-  $\delta$  1. These competitive binding experiments were similar 10 to the binding experiment described above except that non biotinylated lipids are added to each reaction to compete with biotinylated Pea-PI(4,5)P<sub>2</sub> for binding to PLC-  $\delta$  1-PH domain. For example, eight concentrations of PI(3,4,5)P<sub>3</sub> diC4, PI(4,5)P<sub>2</sub> diC4, and Pea-PI(4,5)P<sub>2</sub> were added as 15 competitors to separate wells in addition to 0.2 pmol/well PLC- $\delta$ -1- PH domain, 0.4 pmol/well of Pea-PI(4,5)P<sub>2</sub>, streptavidin donor, and anti-GST acceptor beads; and incubated as previously described. It was determined that Pea-PI(4,5)P<sub>2</sub> was the best competitor with an IC50 value of 140 nM, and the incorrect headgroup PI(3,4,5)P<sub>3</sub> demonstrated the least affinity with an 20 IC50 value of 70 mM. This 500 times better affinity of Pea-PI(4,5)P<sub>2</sub> for PLC- d1-PH domain compared to PI(3,4,5)P<sub>3</sub> is fundamental to the development of a robust assay where one detects the specific PIPn product of a kinase or phosphatase in the presence of excess PIPn substrate.

All of the COMPOSITIONS, METHODS and APPARATUS disclosed 25 and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the COMPOSITIONS, METHODS and APPARATUS and in the 30 steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein

while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

**What is claimed is:**

1. A functionalized phosphoinositide polyphosphate comprising a carbon backbone, a phosphatidylethanolamine head group at carbon position 1, and a PtdInsP<sub>n</sub> head group at carbon position 4.  
5
2. A functionalized phosphoinositide polyphosphate of claim 1, wherein said carbon backbone is selected from the group consisting of 2,3-diacylthreitol, erythritol and a synthetic module.  
10
3. A functionalized phosphoinositide polyphosphate of claim 2, wherein said synthetic module is prepared and coupled with a PtdInsP<sub>n</sub> head group.  
15
4. A functionalized phosphoinositide polyphosphate of claim 3, wherein said PtdInsP<sub>n</sub> head group is selected from the group consisting of (PI, PI(3)p, PI(4)p, PI(5)p, PI(4,5)P<sub>2</sub>, PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, PI(3,4,5)P<sub>3</sub>).
5. A functionalized phosphoinositide polyphosphate of claim 1, further comprising a reporter group.  
20
6. A functionalized phosphoinositide polyphosphate of claim 5, wherein the reporter is selected from the group consisting of a fluorophore, a spin label, biotin, a radio label, a chemiluminescent label, a photophore, a chromophore, a nanogold particle and mixtures thereof.  
25
7. A functionalized phosphoinositide polyphosphate of claim 6, wherein the fluorophore is selected from the group consisting of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein, hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, and X-Rhodamine.  
30

8. A functionalized phosphoinositide polyphosphate of claim 5, wherein the reporter is covalently attached to a free amino group on the phosphatidylethanolamine head group.
- 5 9. A functionalized phosphoinositide polyphosphate of claim 8, wherein the reporter is attached to a free amino group by a linker.
- 10 10. A functionalized phosphoinositide polyphosphate of claim 9, wherein said linker comprises an oligo-polyethylene glycol linker.
11. A functionalized phosphoinositide polyphosphate of claim 9, wherein said linker may include an additional or alternative linker.
12. A functionalized phosphoinositide polyphosphate of claim 11, wherein 15 said additional or alternative linker is selected from the group consisting of a diamino linker, a linker utilizing both phospho- and non-phospho linked spacers, a phosphatidylserine linker and a carboxylic acid linker.
- 20 13. A functionalized phosphoinositide polyphosphate of claim 11, wherein said linker includes aminoalcohols, heteroatom-containing derivatives or branched aminoalcohols.
14. A functionalized phosphoinositide polyphosphate of claim 13, further 25 comprising a diamino linker.
15. A functionalized phosphoinositide polyphosphate of claim 1 selected from the group consisting of Pea-PI, Pea-PI(3)P, Pea-PI(4)P, Pea-PI(5)P, Pea-PI(3,4)P<sub>2</sub>, Pea-PI(3,5)P<sub>2</sub>, Pea-PI(4,5)P<sub>2</sub> and Pea-PI(3,4,5)P<sub>3</sub>.

16. A functionalized phosphoinositide polyphosphate of claim 1, wherein the carbon backbone comprises 2 acyl chains having from 2 to 26 carbons.
- 5 17. A functionalized phosphoinositide polyphosphate of claim 1, wherein the carbon backbone comprises an ether chain in the place of one or both acyl chains.
- 10 18. A functionalized phosphoinositide polyphosphate of claim 1, further comprising one or more phosphate groups that have been chemically modified to stabilize the compound against chemical or enzymatic hydrolysis.
- 15 19. A functionalized phosphoinositide polyphosphate of claim 1, further comprising a polymerizable group.
- 20 20. A method of screening for phosphoinositide-specific binding proteins comprising (a) contacting a functionalized phosphoinositide polyphosphate comprising a carbon backbone, a phosphatidylethanolamine head group at carbon position 1, and a PtdInsP<sub>n</sub> head group at carbon position 4 with a putative phosphoinositide-specific binding protein-containing composition; and (b) measuring binding
- 25 21. A method of claim 20, wherein said functionalized phosphoinositide polyphosphate is attached to a surface.
- 30 22. A method of claim 21, wherein said surface is selected from the group consisting of plates, beads, liposomes, nitrocellulose and metals.
23. A method of claim 21, wherein said attaching a functionalized phosphoinositide polyphosphate is selected from the group consisting of streptavidin and NHS activation.

24. A method of claim 20, wherein said functionalized phosphoinositide polyphosphate is selected from the group consisting of Pea-PI, Pea-PI(3)P, Pea-PI(4)P, Pea-PI(5)P, Pea-PI(3,4)P<sub>2</sub>, Pea-PI(3,5)P<sub>2</sub>, Pea-PI(4,5)P<sub>2</sub> and Pea-PI(3, 4,5)P<sub>3</sub>.

5

25. A method of claim 20, wherein said functionalized phosphoinositide polyphosphate further comprises a reporter.

10 26. A method of claim 25, wherein said reporter is selected from the group consisting of a fluorophore, a spin label, biotin, a radio label, a chemiluminescent label, a photophore, a chromophore, a nanogold particle and mixtures thereof.

15 27. A method of claim 20, wherein said method comprises an assay selected from the group consisting of an *in vitro* enzyme assay, an *in vitro* agonist assay, an *in vitro* antagonist assay, a cell-based assay, a lipid kinase activity assay, a protein kinase activity assay, a lipid phosphatase activity assay, a protein phosphatase activity assay, a phospholipase assay and a phosphatase activity assay.

20

28. A method of claim 20, wherein said phosphoinositide-specific binding protein is selected from the group consisting of protein kinases, phosphoinositide kinases, phosphoinositide binding proteins, inositol phosphate binding proteins, lipid phosphatases and phospholipases.

25

29. A method of identifying compositions that affect phosphoinositide recognition or signaling at a cell membrane comprising (a) contacting a functionalized phosphoinositide polyphosphate comprising a carbon backbone, a phosphatidylethanolamine head group at carbon position 1, and a PtdInsP<sub>n</sub> head group at carbon position 4 with a composition that putatively affects phosphoinositide recognition or signaling; and (b) measuring recognition and signaling.

30

30. A method of claim 29, wherein said composition that putatively affects phosphoinositide recognition or signaling comprises a compound selected from the group consisting of a chemical, a natural product, and a synthetic compound.

5

31. A method of claim 29, wherein said composition that putatively affects phosphoinositide recognition or signaling comprises a compound selected from the group consisting of lipid phosphatases and phospholipases.

10

32. A method of claim 28, wherein said method comprises a cell-based assay.

15

33. A functionalized phosphoinositide polyphosphate comprising Pea-PI, Pea-PI(3)P, Pea-PI(4)P, Pea-PI(5)P, Pea-PI(3,4)P<sub>2</sub>, Pea-PI(3,5)P<sub>2</sub>, Pea-PI(4,5)P<sub>2</sub> or Pea-PI(3, 4,5)P<sub>3</sub>.

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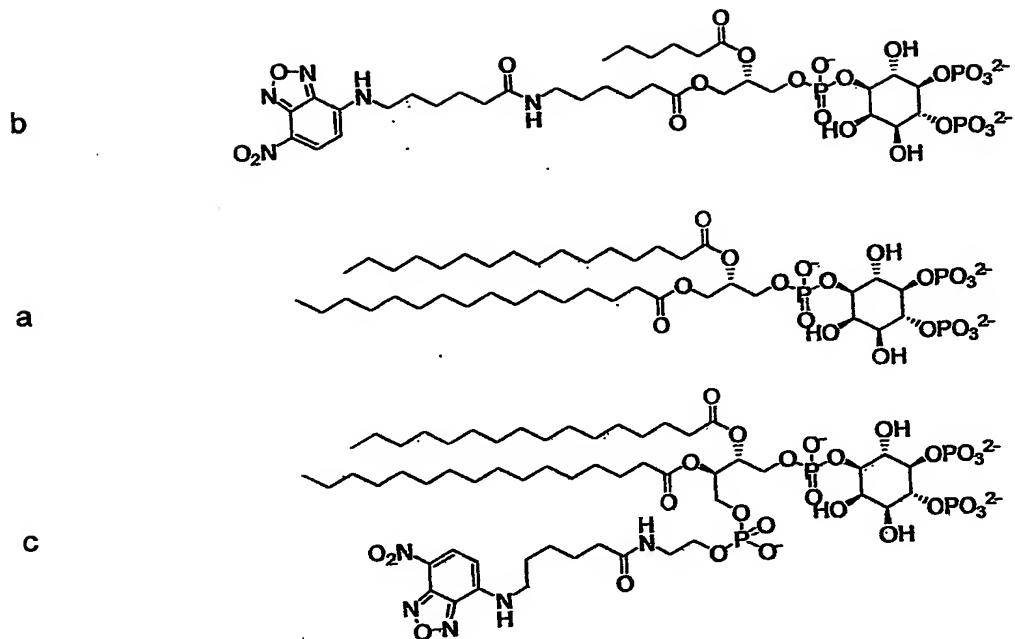


FIG. 1

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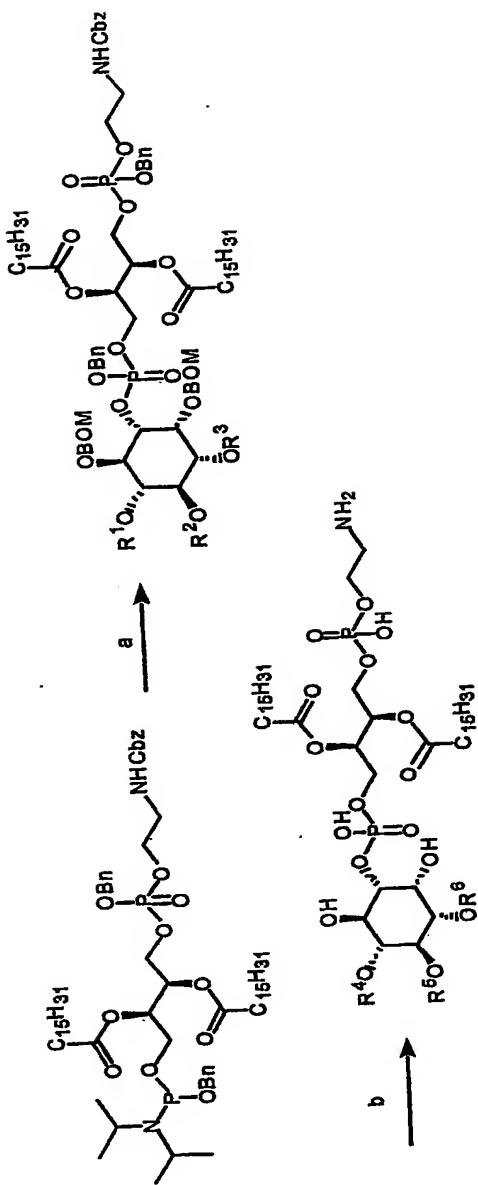


FIG. 2

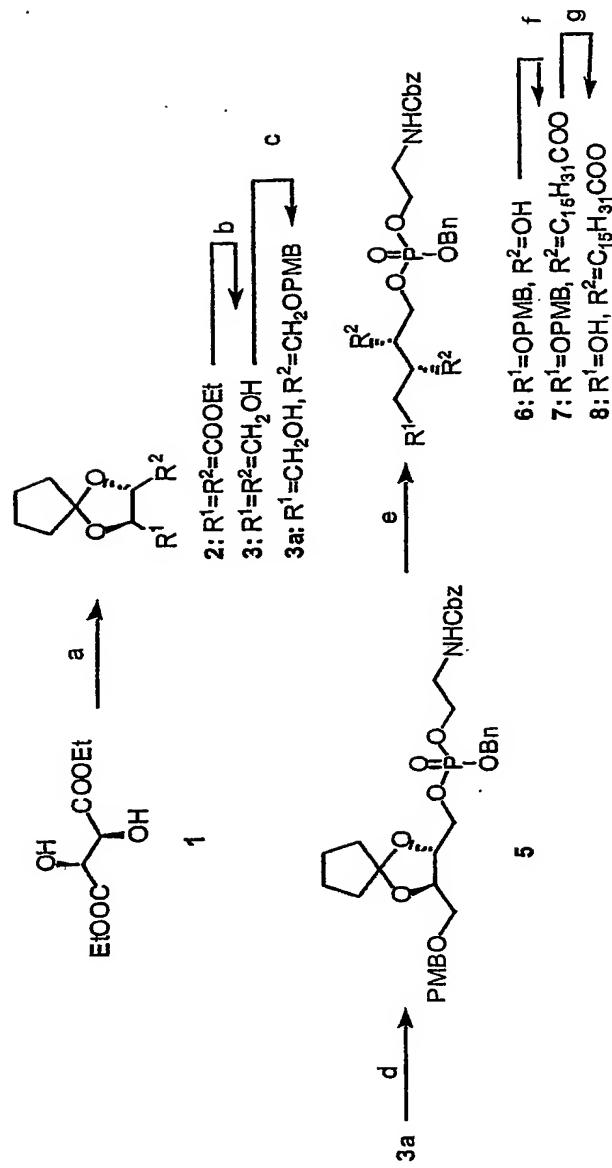


FIG. 3

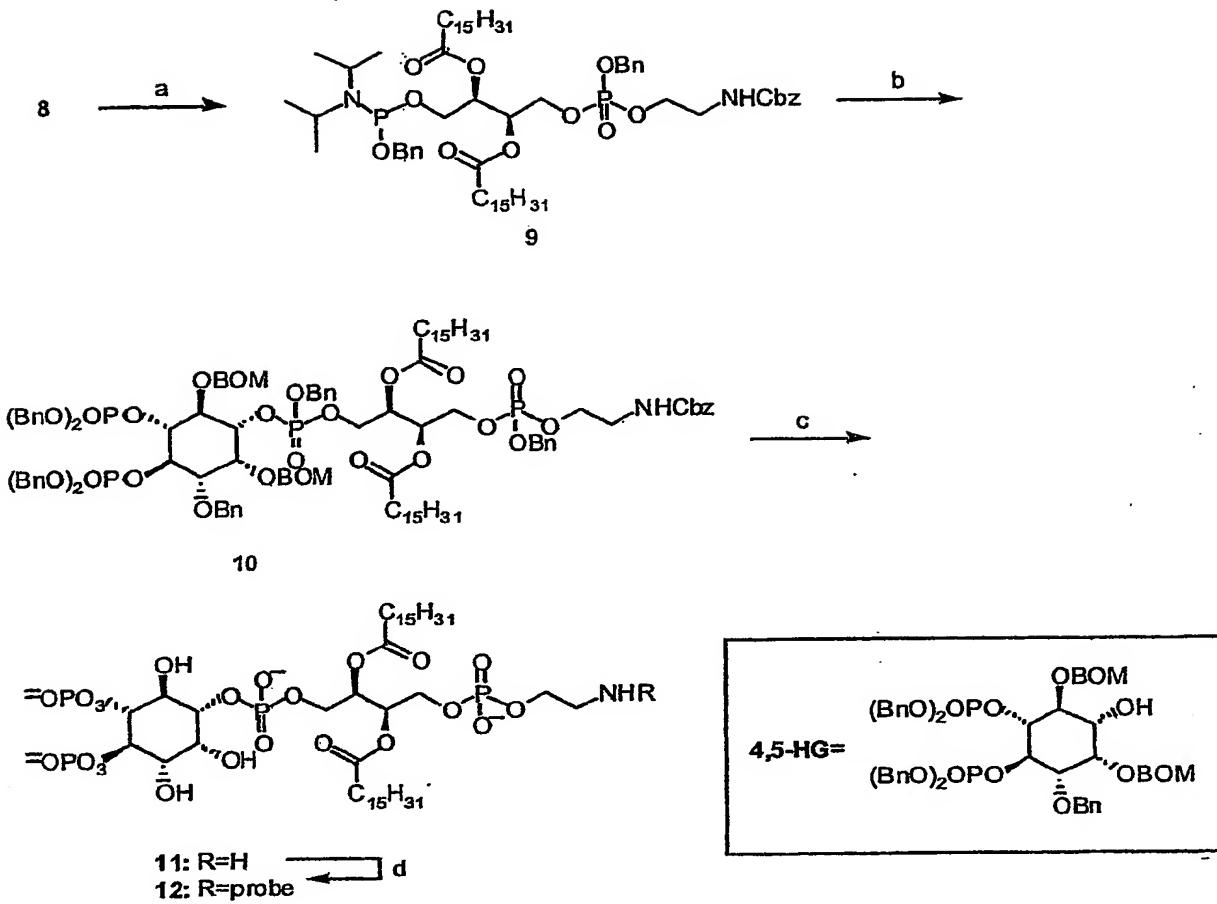
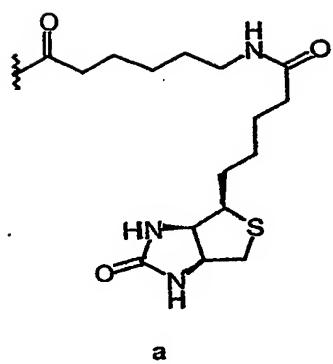
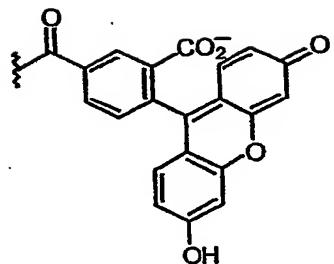


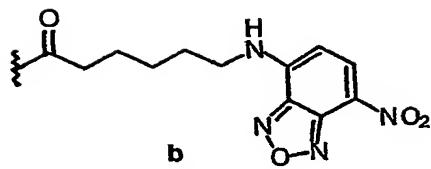
FIG. 4



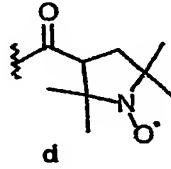
a



c



b



d

FIG. 5

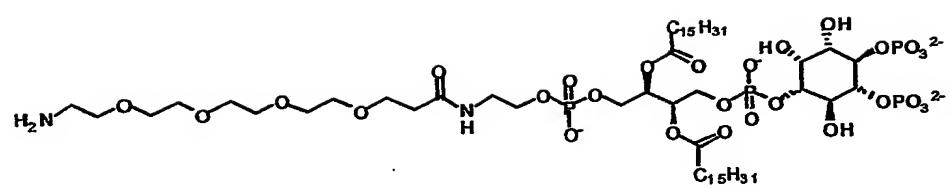


FIG. 6

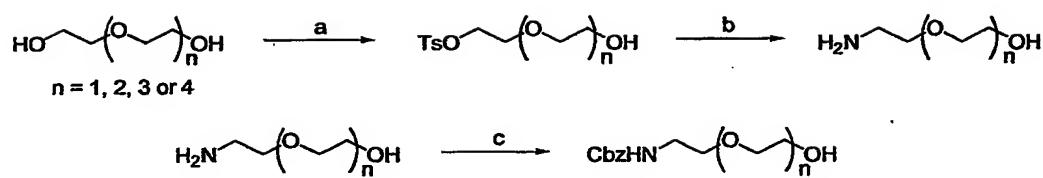


FIG. 7

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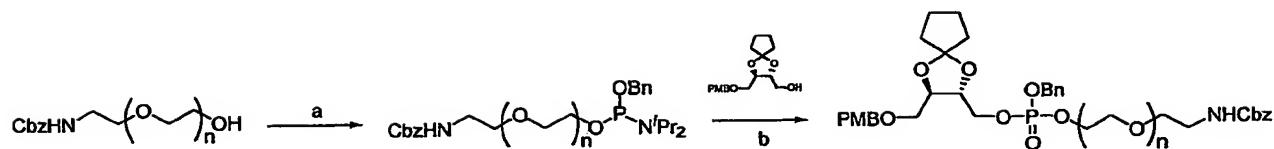


FIG. 8

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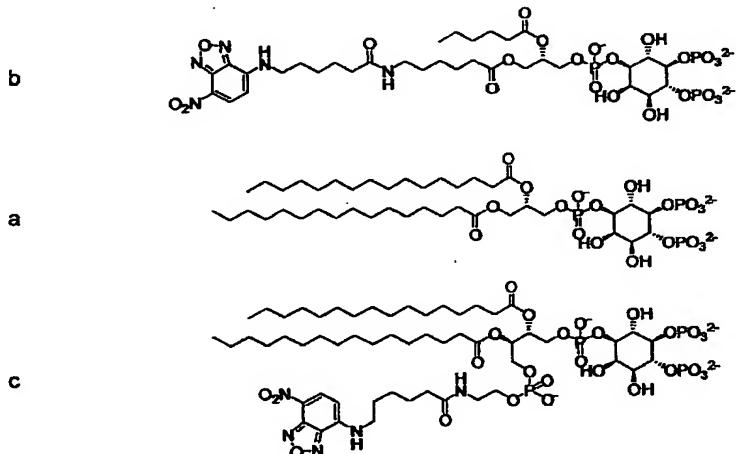
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[Continued on next page]

(54) Title: HYBRID PHOSPHOINOSITIDE PHOSPHOLIPIDS: COMPOSITIONS AND USES



WO 2003/082903 A3

(57) Abstract: The methods and compositions disclosed synthesis of a novel class of "two-headed" phospholipid-phosphoinositide hybrids possessing a carbon backbone, such as 2,3-diacylthreitol, erythritol or a synthetic module. The second phospholipid head group allows introduction of a biochemical or chemical moiety in a position orthogonal in space to those occupied by the phosphoinositide head group and the two acyl chains. The diacyl moieties allow for the incorporation of Pea-PIP<sub>2</sub> into a lipid bilayer, while the PtdIns(4,5)P2 moiety in the aqueous layer is specifically recognized by lipid binding proteins. In alternative embodiments of the invention, reporters, for example biotin, fluorophores and/or spin labels, are attached to the free amino group of the head groups of such molecules to specifically target the reporters to the lipid-water interface. Figure (1) illustrates (at c) an exemplary hybrid lipid of the present invention that possess a phosphatidylethanolamine (PE, or Pea) head group at the 1-position and a PtdIns(4,5)P2 head group at the 4-position.



European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US03/09858

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/00, 1/42, 1/48; G01N 33/58  
US CL : 435/4, 21, 7.1, 15

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 21, 7.1, 15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Non-Patent Literature

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST:phosphotidylinositide polyphosphate, carbon backbone, phosphatidylethanolamine, phosphotidylinositides head group, phosphoinositide-specific protein binding method

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 20020028477 A1 (GOUELI et al) 07 March 2002, see entire document.	1-33
A/P	US 20030100028 A1 (DREES et al) 29 May 2003, see entire document.	1-33

Further documents are listed in the continuation of Box C.  See patent family annex.

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"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
28 NOVEMBER 2003	06 FEB 2004

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